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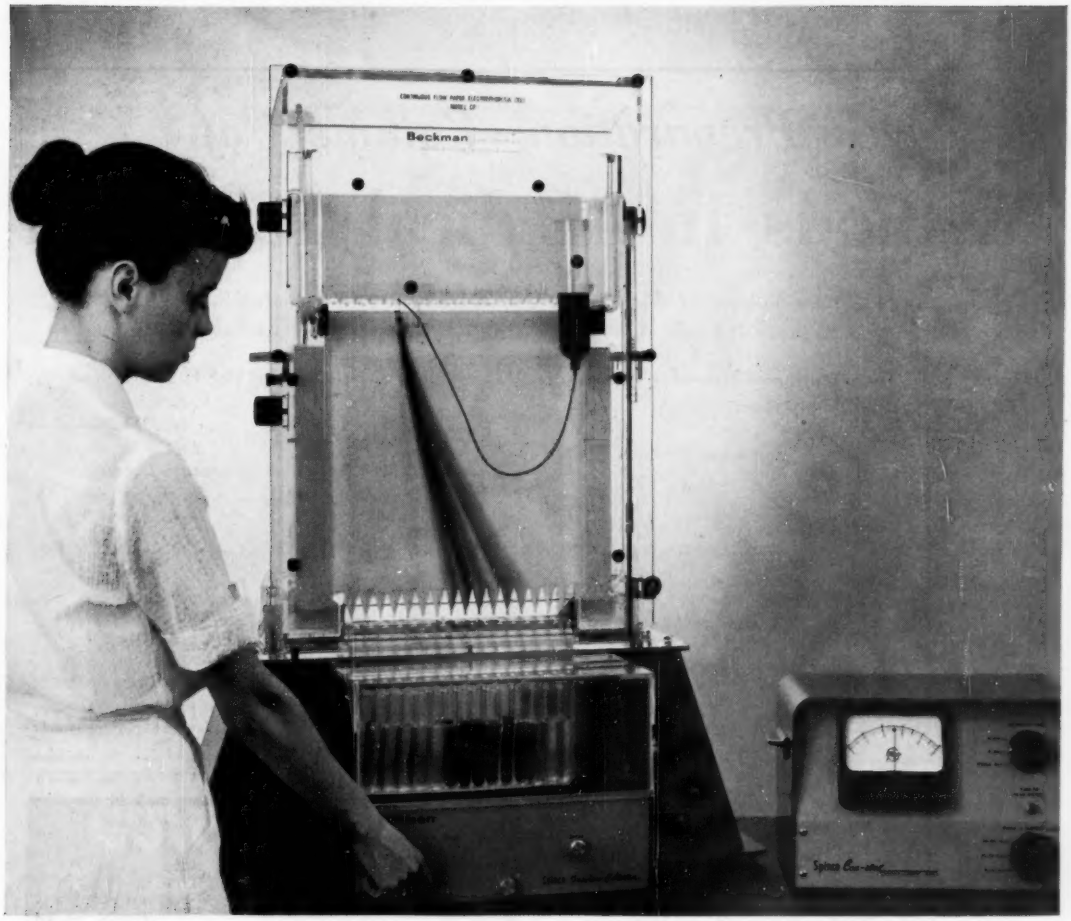

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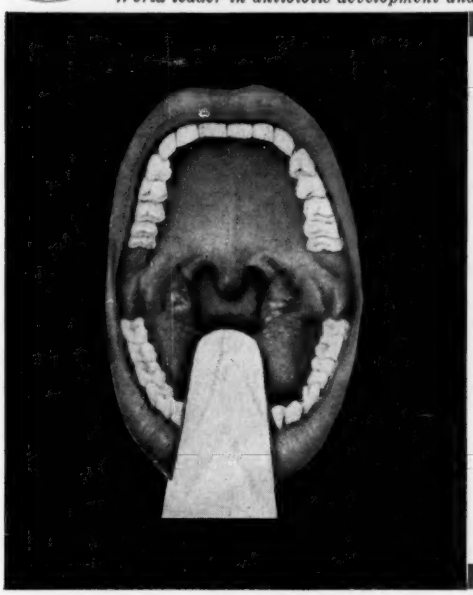

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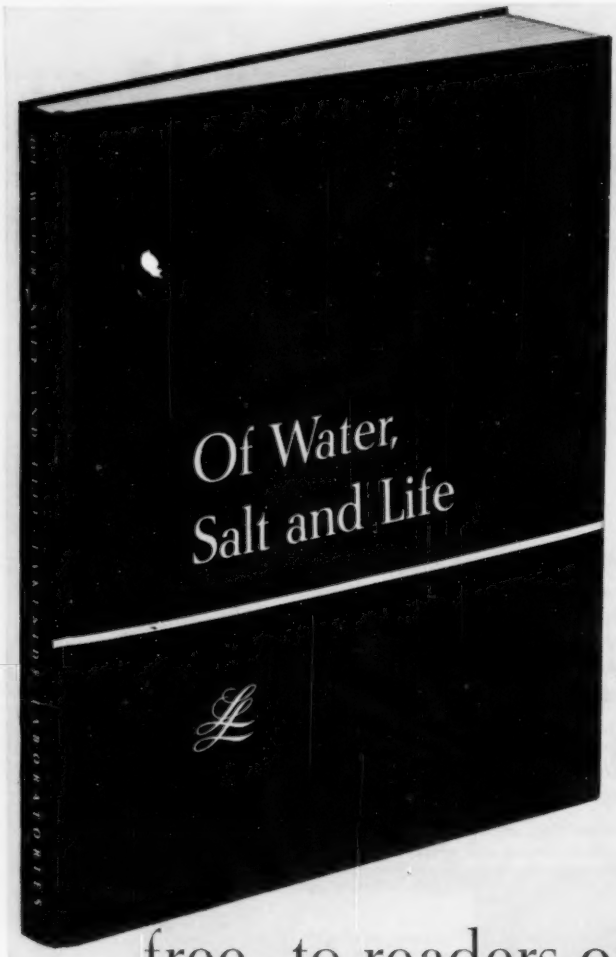
1. Carter, C. H., and Maley, M. C.: Antibiotics Annual 1956-1957, New York, Medical Encyclopedia, Inc., 1957, p. 51.



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ELECTROPHORETIC STUDIES OF RED CELL EXTRACTS OF STORED BLOOD[1]

By EDWARD R. BERRY and ALFRED CHANUTIN with the assistance of CLAUDE A. SMITH

(From the Department of Biochemistry, School of Medicine, University of Virginia, Charlottesville, Va.)

(Submitted for publication March 5, 1956; accepted October 10, 1956)

It has been shown that red cells of blood collected in acid-citrate-dextrose (ACD) undergo changes in their dimensions, osmotic fragility, permeability and metabolism during storage at 4° C (1). Information concerning the effect of storage on the physical and chemical states of soluble red cell proteins, particularly hemoglobin, is not available. Such data may be of interest in view of the marked changes observed in the mobility and concentration of several proteins during the storage of plasma (2). This paper presents evidence from electrophoretic analyses that the concentration of components of extracts of red cells is changed during storage of blood.

METHODS

Healthy male medical students, about 21 years of age, and anemic hospitalized patients served as donors. Sterile precautions were taken in the collection, storage and sampling of the blood. As a rule, 50 ml. of blood was drawn from each individual before breakfast and collected in a 125-ml. cotton-plugged, sterile, Pyrex Erlenmeyer flask containing 12.5 ml. ACD (NIH Sol. B). Sterilized ACD-inosine or ACD-adenosine solutions were also used in the initial collection of blood. Stored blood was supplemented by calculated amounts of nucleoside in a sterilized saline solution. Phenergan® (phenothiazine, 10-(2-dimethylaminopropyl)-hydrochloride), dissolved in saline, was sterilized by filtration through an ultra-fine fritted Pyrex glass disc, and the required amounts were added to the sterile ACD solution. After removing a 5-ml. aliquot of blood for analysis, the remaining blood was stored at 4° C. It was necessary to withdraw 10-ml. aliquots for analysis if there was an excessive loss of red cells during the washing procedures or whenever the hematocrit values of the blood were low.

The blood sample was centrifuged, the plasma and the top cellular layer were removed; the cells were washed and lysed at room temperature and clarified by centrifugation in the refrigerator, according to Drabkin's procedure (3). The first step involved washing once with 0.9 per cent NaCl and three times with a 1.2 per cent NaCl-0.0025 M $AlCl_3$ mixture. Clarified solutions were examined under oil immersion with a phase microscope without observing stroma or stroma filaments. The hemoglobin concentration of the clarified red cell extract was determined (4) and an appropriate volume was diluted with distilled water to yield 5 ml. of a 1.2 or 1.4 per cent hemoglobin solution. This solution was dialyzed, in the cold, against 0.05 M sodium cacodylate-cacodylic acid buffer (pH 6.5) which was changed three times during a 24-hour period. Electrophoresis was done in a Klett Model of the Tiselius apparatus using a microcell of 2-ml. capacity. The temperature was maintained at $2.0 \pm 0.01°$ C. Electrophoresis was allowed to proceed for 88 minutes with an open anode vessel and the boundaries were compensated (without interrupting the current) to the center of the cell; the run was continued for 30 additional minutes with a closed anode vessel. Photographs were taken by Longsworth's scanning technique (5) using CTC panchromatic plates and a Wratten No. 25 filter. These patterns were better defined than those obtained with the Philpot-Svensson cylindrical lens method. The ascending patterns were analyzed by dropping lines at the minima between boundaries (6), and where there was no well-defined minimum the same relative position of the partitioning line was maintained. The patterns of the descending limb were not analyzed because the boundaries were poorly separated.

The tests for osmotic fragility were similar to those recommended by Schales (7).

Adenosine and inosine were purchased from the Nutritional Biochemicals Corporation and from the Schwarz Laboratories, Inc., and the Phenergan® was obtained from Wyeth, Inc.

RESULTS

Conditions for electrophoresis of red cell extracts

Aliquots of a red cell extract were analyzed by electrophoresis in cacodylate buffer solutions (pH 6.5) with molar concentrations of 0.1, 0.075, 0.05, and 0.025. Under these experimental conditions, a single boundary is obtained with the 0.1 M buffer. The pattern obtained with 0.075 M buffer shows a well-defined small and a large component

[1] This investigation was supported by the Medical Research Development Board, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-160.

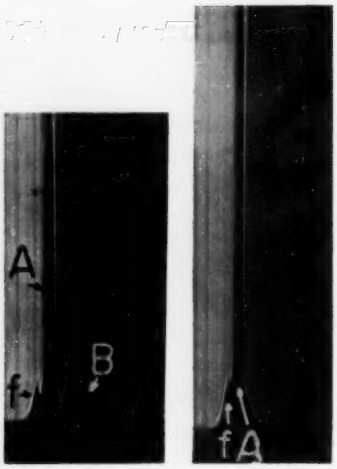

FIG. 1. ELECTROPHORETIC PATTERNS OF RED CELL EXTRACTS

(a) Freshly drawn blood.
(b) ACD blood stored 50 days.

and a small shoulder on the leading edge of the front component. Three well-defined boundaries (f, A and B) are observed in the 0.05 M buffer (Figure 1, a). The pattern of the 0.025 M buffer aliquot was difficult to analyze because of excessive sharpening of the boundaries. Aliquots of a red cell extract were adjusted to 1.25 per cent total hemoglobin and were analyzed at pH 6.4, 6.5 and 6.6 without showing significant differences in the distribution of the components. In addition, solutions varying in concentration from 0.75 to 1.75 per cent hemoglobin were analyzed at pH 6.5 and the results were similar to those observed with the 1.25 per cent solution. As a result of these preliminary experiments, the red cell extracts were routinely analyzed in a 0.05 M cacodylate buffer at pH 6.5 between 1.25 and 1.5 gm. per cent hemoglobin. Under these conditions, a steady electrophoretic state, as defined by Hoch (8) and by Nichol (9), is obtained.

ACD controls

The patterns of red cell extracts of freshly drawn ACD bloods from male students were analyzed by the Philpot-Svensson method. The concentration for component B was determined as per cent of the total area for the three components. The average per cent concentration of component B for 12 subjects was 24.5 with minimum and maximum values of 22.0 and 26.7, respectively. The Longsworth scanning procedure gives values which are 6 to 10 per cent greater than by the Philpot-Svensson method.

Effect of storage

ACD and ACD plus Phenergan®, inosine and adenosine. The effects of Phenergan®, inosine and adenosine on the concentration of component B and on the degree of hemolysis in 0.6 and 0.85 per cent NaCl were determined on a single sample of blood in order to evaluate their relative effects (Figures 2, 4, 6, 7). A unit of blood (480 ml.), obtained from a healthy young man (E. S.), was collected in 120 ml. ACD (NIH Sol. B) and aliquots were supplemented with the test materials.

The values for the percentage distribution of boundaries f, A and B are subject to errors inherent in the Tiselius-Kabat line-dropping procedure (6) and in the dissymmetry of the boundaries. Inspection of pattern b in Figure 1 indicates that component B is not present. Nevertheless, the line-dropping procedure makes it necessary to designate about 10 per cent of the total pattern as component B, when in reality most, if not all, of this area represents the trailing foot of component A. In presenting results for changes in terms of per cent of the original component B concentration, values of 30 to 40 per cent may be

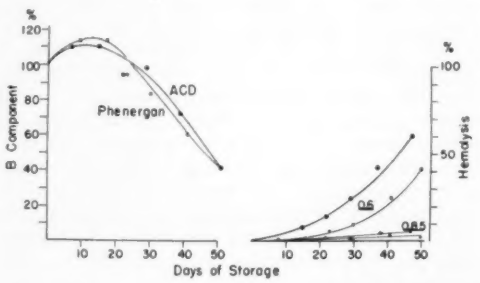

FIG. 2. EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH PHENERGAN® (0.4 MM PER L. BLOOD) ON COMPONENT B CONCENTRATION AND ON OSMOTIC FRAGILITY

The total hemoglobin concentration of the red cell extracts in this and subsequent experiments was about 1.2 per cent. The figures 0.6 and 0.85 in this and other figures refer to the percentage concentration of NaCl.

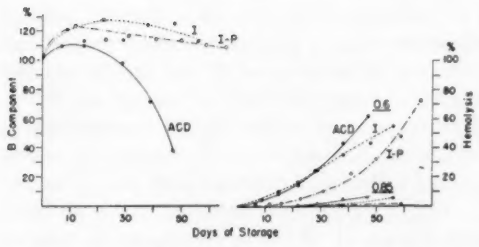

FIG. 3. EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH INOSINE (I) AND INOSINE AND PHENERGAN® (I–P)

Inosine (2,500 μM per 100 ml. RBC) and Phenergan® (0.4 mM per L. blood) were added immediately after collection of blood (E. S.).

encountered for patterns having practically none of this material present.

Phenergan®. The observation of Schales (7) that Phenergan® retarded the rate of increase of osmotic fragility of red cells in 0.6 per cent NaCl of ACD stored blood is confirmed, as shown in Figure 2. The changes in the concentrations of component B in stored ACD and ACD-Phenergan® blood are about the same. There appears to be no relationship between the osmotic fragility and the distribution of the components of the red cell extracts in these experiments.

A study of the effect of another phenothiazine derivative, chloropromazine, was discontinued because of its hemolytic properties.

Inosine. Donohue, Finch, and Gabrio (10) implicated inosine, the product of adenosine deamination, as the agent which is effective in erythrocyte preservation, and Gabrio, Donohue, Huennekens, and Finch (11) demonstrated that this nucleoside prolonged the viability of the red cell. Data plotted in Figure 3 show the changes in the concentration of the component B in (a) ACD control, (b) inosine and (c) inosine-Phenergan®-supplemented aliquots of a single blood during storage. The concentration of component B remains elevated for more than 60 days in the inosine-containing aliquots. In contrast, the values for this component in the ACD aliquot increase during the first few weeks and subsequently decrease at a comparatively rapid rate. The osmotic fragility of the inosine-Phenergan® red cells in 0.6 per cent NaCl is much lower than that of the inosine and ACD samples. The values for the per cent hemolysis after 47 days of storage of aliquots a, b, and c are 61, 43 and 28, respectively.

Inasmuch as the addition of inosine plus Phenergan® to ACD blood had a profound effect on maintaining a high component B concentration and low osmotic fragility during storage, additional data were obtained with samples of blood from three different donors (Figure 4). Considerable individual variations are noted in these experiments. The component B concentrations remained elevated during periods of about 40 to 60 days. It will be noted that the extent of hemolysis in 0.6 per cent NaCl is related to the time that the concentration of component B remains elevated.

Experiments were designed to determine the influence of varying concentrations of inosine on the electrophoretic patterns of stored blood. In Figure 5, results are shown for three individual ACD bloods supplemented with 1) 2,500 μ moles, 2) 1,250 μ moles, and 3) 625 μ moles inosine per 100 ml. of red cells. The data for bloods 2 and 3 indicate that the rate of decrease in component B concentration is greater than that of ACD blood (Figure 2). After storage for 48 days, 2,500 μ moles and 1,250 μ moles of inosine per 100 ml. of red cells were added to bloods 3 and 2, respectively. The results are striking since the concentration of component B of blood 3 is elevated to the control level and remains high during the period of observation. The value for blood 2 is also elevated from about 40 to 85 per cent but

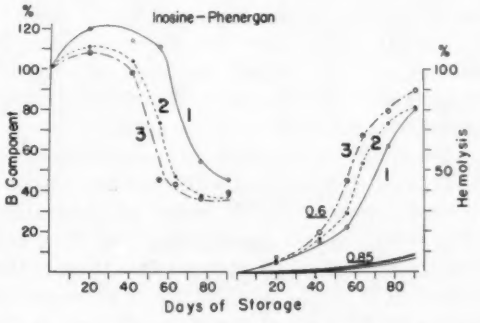

FIG. 4. EFFECT OF STORAGE OF ACD SUPPLEMENTED WITH INOSINE PLUS PHENERGAN®

Curves 1, 2 and 3 represent data for bloods from three healthy male donors. Concentrations of inosine and Phenergan® are the same as those in the previous experiment (Figure 3).

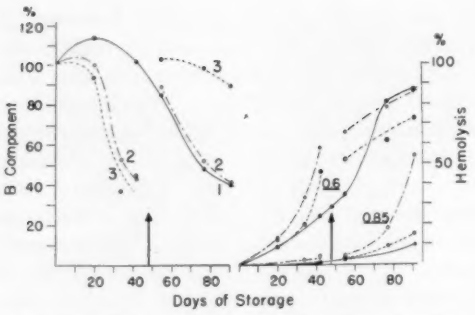

FIG. 5. EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH VARYING AMOUNTS OF INOSINE

Inosine was added to three different bloods when drawn. Sample 1, 2,500 μ moles, sample 2, 1,250 μ moles, and sample 3, 625 μ moles per 100 ml. RBC. After samples 2 and 3 were stored for 48 days, 1,250 and 2,500 μ moles inosine per 100 ml. RBC were added to the respective bloods.

subsequently decreases at the same rate observed for blood 1.

The values for the per cent hemolysis in 0.6 per cent NaCl of red cells of blood 1 are markedly increased after about 50 days of storage and practically all the cells are hemolyzed after 90 days. The osmotic fragilities of the red cells of bloods 2 and 3 are similar but are appreciably greater than those of blood 1, during the first 40 days of storage. The additional supplement of 2,500 μ moles of inosine to blood 3 prevents the marked increase in osmotic fragility. On the other hand, addition of a smaller amount of inosine to blood 2 has a temporary effect. The increased values for the per cent hemolysis of red cells of blood 2 in isotonic saline after the second addition of inosine probably reflect the lack of optimal amounts of nucleoside, which is undoubtedly concerned with the integrity of the stroma.

Adenosine. Component B concentration of ACD blood supplemented with adenosine remains elevated during a 60-day period of observation (Figure 6). It will also be noted that these red cells are more readily hemolyzed than those of the control ACD blood. The addition of adenosine or inosine to ACD blood stored for 22 days is responsible for maintaining a prolonged, elevated component B concentration and for a decreased osmotic fragility.

Effect of temperature. Three aliquots of the ACD blood were stored at 4°, 20° and 37° C. The results in Figure 7 show that the storage temperature has a pronounced effect on the concentration of component B and on the osmotic fragility. After 10 hours of storage at 37° C, about 60 per cent of the original concentration of component B disappears. A similar but not as marked a change is observed after storage at 20° C. Osmotic fragility is increased within 48 hours after storage at 37° C; a corresponding value is observed at the end of 8 days of storage at 20°.

Bloods from anemic patients. The data in Figure 8 show the changes in component B concentrations during storage of 13 bloods from patients with anemia of primary and secondary origin. The rapid decrease in values for red cell extracts from patients 1 to 5 is unusual and striking. The data for patients 6 to 8 fall within the range observed for control ACD blood during the first 30 days of storage. The values for cases 9 and 10 remain at a constant level during the period of observation. An unusually high concentration (36 per cent) of component B values for the erythrocytes of patients 11, 12 and 13 is

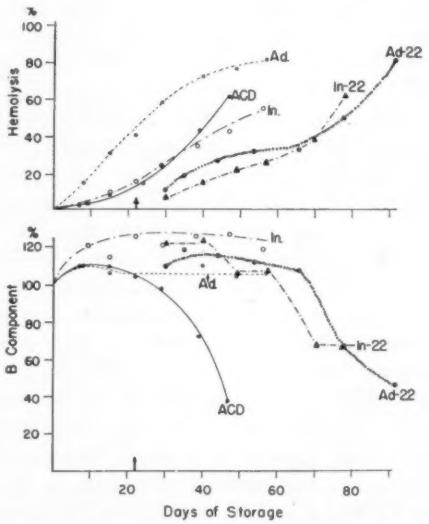

FIG. 6. EFFECT OF STORAGE OF ACD BLOOD SUPPLEMENTED WITH ADENOSINE (Ad) AND WITH INOSINE (In)

At 0 days, a suspension of adenosine (2,500 μ moles per 100 ml. RBC) was added to the blood (E. S.). After storage of ACD blood for 22 days, adenosine (2,500 μ moles) was added and incubated for 1 hour at 37° C. Inosine was added to blood under the same conditions.

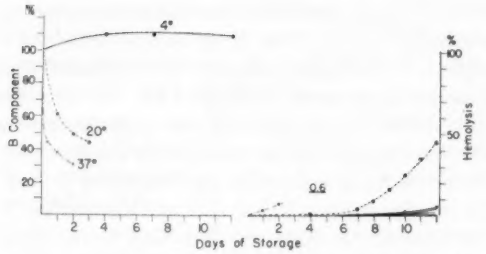

FIG. 7. EFFECT OF STORAGE OF ACD BLOOD (E. S.) AT 4°, 20° AND 37° C

striking. This is particularly true for the case of lymphocytic leukemia in which the initial concentration of component B (17.7 per cent) was the lowest value observed in these experiments.

The osmotic fragilities of the red cell in 0.6 per cent NaCl from these anemic patients tend to be consistently below the values observed for red cells from healthy individuals. The lack of any relationship between the behavior of component B and osmotic fragility is apparent in this group.

DISCUSSION

Evidence for the heterogeneity of hemoglobin has been presented by a number of investigators. Geiger (12) first demonstrated the presence of two different hemoglobins in the red cells of a number of species. He found that the hemoglobins could be separated most effectively if a red cell extract was subjected to cataphoresis at a low ionic strength. Additional evidence was provided by solubility studies (13), by electrophoresis (14–16), by oxygen capacity measurements (17, 18), by chromatographic separation (19–21), and by stability to alkaline denaturation (22–27). Schapira, Dreyfus, and Kruh (28) were able to prove the existence of at least two hemoglobins after treating extracts of Fe^{59}-labelled red cells of a single blood by alkali denaturation, electrophoresis, paper chromatography and alumina chromatography. In addition, globin, prepared from hemoglobins of several species, was found to consist of two components (29, 30). Data presented in this paper show the presence of at least two pigmented boundaries or components in red cell extracts which are assumed to be hemoglobins.

The identity of each of the three boundaries observed in the electrophoretic patterns of red cell extracts is not known. The fast moving boundary (component f) can be observed as a colorless material and probably represents the concentration gradient of the cacodylic acid mixture. Experiments to test this were conducted by comparing the areas of component f after electrophoresing aliquots of a red cell hemolysate in 0.05 M buffer into 0.047, 0.050 and 0.053 molar cacodylate buffers (pH 6.5) in the ascending limb. The areas of this boundary were smaller in the 0.047 and greater in the 0.053 molar solutions, which indicates that this boundary is due to the cacodylate ion. Components A and B are deeply pigmented boundaries which are assumed to be hemoglobins.

Derrien and Reynaud (14) observed heterogeneity in 0.1 M cacodylate (pH 6.5) if electrophoresis was continued for a sufficiently long period. In the present experiments, a single boundary was obtained for red cell extracts when electrophoresis was done at pH 6.5 in 0.1 M cacodylate buffer for two hours and heterogeneity was encountered in 0.075 M and lower concentrations. Shavit and Breuer (16) believe that hemoglobin dissociates into several different elementary units at low ionic strengths. During the storage of

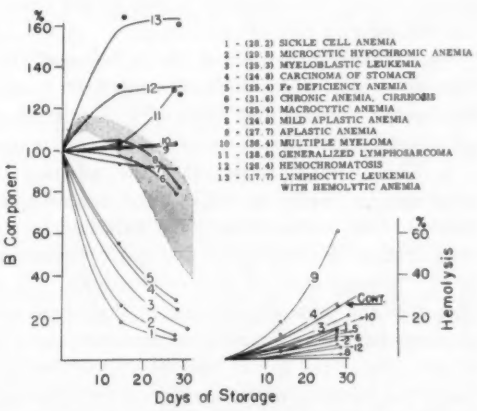
1 - (20.2) SICKLE CELL ANEMIA
2 - (22.5) MICROCYTIC HYPOCHROMIC ANEMIA
3 - (19.3) MYELOBLASTIC LEUKEMIA
4 - (24.8) CARCINOMA OF STOMACH
5 - (23.4) Fe DEFICIENCY ANEMIA
6 - (31.6) CHRONIC ANEMIA, CIRRHOSIS
7 - (23.4) MACROCYTIC ANEMIA
8 - (24.8) MILD APLASTIC ANEMIA
9 - (27.7) APLASTIC ANEMIA
10 - (30.4) MULTIPLE MYELOMA
11 - (30.6) GENERALIZED LYMPHOSARCOMA
12 - (20.6) HEMOCHROMATOSIS
13 - (17.7) LYMPHOCYTIC LEUKEMIA WITH HEMOLYTIC ANEMIA
B Component
Hemolysis
CONT.
Days of Storage

FIG. 8. EFFECT OF STORAGE OF BLOOD (ACD) OF PATIENTS WITH ANEMIA

The patterns for these bloods were obtained with the Philpot-Svensson cylindrical lens method. The numbers in parentheses represent the 0 day concentration of component B; these values are designated as 100 per cent. The stippled area represents the range of values for component B concentration of 12 bloods from healthy young men.

blood, electrophoretic analyses of red cell extracts under identical conditions show that the component B concentration decreases and component A concentration increases. After addition of nucleosides to stored blood, the component B concentration is appreciably increased. It must be concluded that these effects are not due to changes in ionic concentrations.

The degree of hemolysis of red cells of ACD blood washed with 1.2 per cent NaCl-0.0025 M $AlCl_3$ solution increased progressively with the time of storage. The wash solutions and the red cell extracts of bloods stored for 20 and 50 days were studied by electrophoresis. It was observed that the wash solutions contained components f and A while the respective red cell extracts contained components f, A and B. To explain these results it may be assumed that the oldest red cells are hemolyzed during washing and the mobility of component B has increased to that of component A.

Crystallized hemoglobin was prepared by the two methods (3, 31) described by Drabkin. In both cases, only components A and f were observed in the electrophoretic patterns of these crystals. The absence of component B may be accounted for by assuming that this material was altered during crystallization so that its net charge was the same as that of component A.

As a working hypothesis, it is believed that changes in the concentration of component B during storage of blood may serve as an indication of red blood cell viability. Gabrio and her co-workers (11, 32–34) have shown that the addition of adenosine or inosine to ACD blood causes a reversal of the "storage lesion," as indicated by the regeneration of the organic phosphate esters and by the preservation of the erythrocytes. It is shown in the present experiments that these nucleosides have been found to replete component B of red cells stored for 21 and 48 days. Furthermore, the addition of these nucleosides to freshly drawn blood is responsible for an elevation and a prolonged maintenance of component B concentration. According to Gabrio, Stevens, and Finch (35), red cells stored at 37° in ACD maintain viability for one or two days. The rapid decrease in component B concentration during the first 24 hours at 20° and 37° parallels the *in vivo* studies. It is reported that sickle cells survive from 15 to 60 days after transfusion into a normal individual (36). The defect in the sickle cell is reflected by a rapid decrease of component B in blood cells of patient 1 (Figure 8). An excellent correlation exists between the post-transfusion survival of red cells, as recorded in the literature, with the changes observed for component B of red cells. *In vivo* survival studies and electrophoretic analyses on the same red cells must be conducted before a definite correlation is established.

Sack, Gibson, and Buckley (37) have shown that there is a fair correlation between osmotic resistance in 0.6 per cent NaCl and *in vivo* survival of red cells. It would undoubtedly be important to understand the role of stroma integrity in red cell preservation. Schales (7) found that Phenergan®, a phenothiazine derivative, decreased the rate of development of the increased fragility of red cells during storage of ACD blood. Chaplin, Crawford, Cutbush, and Mollison (38) demonstrated that a related phenothiazine compound (RP 3300) had a similar effect but did not increase the ability of the red cell to survive after transfusion. The deterioration and increased permeability of red cells during storage has been attributed to the loss of cholinesterase, which could presumably be prevented by Phenergan® (39). In the present study, Phenergan® depressed the osmotic fragility of stored erythrocytes without affecting the rate of disappearance of component B of stored ACD blood. In the presence of Phenergan® and inosine, both osmotic resistance and component B concentration of stored red cells are maintained for an appreciable time at levels approximating freshly drawn blood. After this blood is stored for a long period, the concentration of component B is maintained at a high level but the osmotic fragility is markedly increased in 0.6 per cent NaCl. If it is assumed that the viability of the stored red cell is dependent on maintaining the integrity of its physical, chemical and metabolic characteristics, it is possible that Phenergan® and inosine may be useful in blood preservation.

SUMMARY

Aqueous red cell extracts prepared from human blood were analyzed by electrophoresis. Three boundaries (f, A and B), listed in the order of

decreasing mobility, were observed in a cacodylate buffer at 0.05 M concentration and pH 6.5. The concentration gradient of the buffer salt is responsible for the f boundary while components A and B appear to be hemoglobins. Component B represents about 25 per cent of the total pattern area of red cell extracts from freshly drawn blood of healthy males.

During storage of ACD and ACD plus Phenergan® bloods, the component B concentrations decrease at the same rate after about 30 days. Osmotic fragility in 0.6 per cent NaCl is lower in the Phenergan®-supplemented blood.

The addition of adenosine (2,500 μ moles per 100 ml. RBC), inosine (2,500 μ moles per 100 ml. RBC) or inosine plus Phenergan® (0.4 mM per liter of blood) is responsible for maintaining component B concentrations at elevated levels during 50 to 60 days of storage. The osmotic fragility was lowest in the presence of inosine plus Phenergan®.

Small amounts of inosine (1,250 and 625 μ moles per 100 ml. RBC) added to ACD blood appear to increase the rate of disappearance of component B during storage.

Addition of adenosine or inosine to ACD blood, stored for 21 or 48 days, causes the repletion of component B.

A marked decrease in component B concentration was observed within the first 24 hours of storage of ACD blood at 20° and 37° C.

Component B concentrations of red cells from patients with anemia showed wide differences during storage.

The component B concentration may serve as an indicator for judging the viability of red cells. The importance of osmotic resistance as a factor in red cell preservation cannot be assessed.

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ANNOUNCEMENT OF MEETING

The 49th Annual Meeting of the American Society for Clinical Investigation will be held in Atlantic City, New Jersey, on Monday, May 6, 1957, with headquarters at the Chalfonte-Haddon Hall. The scientific session will begin at 9 A.M. at the Steel Pier Theater.

THE BIOSYNTHESIS OF THE FATTY ACIDS OF THE PLASMA OF MAN. I. THE FORMATION OF CERTAIN CHROMATOGRAPHICALLY SEPARATED HIGHER FATTY ACIDS OF THE MAJOR LIPIDE COMPLEXES FROM ACETATE-1-C^{14} [1]

By S. R. LIPSKY, A. HAAVIK, C. L. HOPPER, and R. W. McDIVITT with the technical assistance of BARBARA M. MOSSBERG

(From the Department of Medicine, Yale University, New Haven, Conn.)

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One of the outstanding difficulties precluding the use of radioactive tracers for a detailed study of the dynamics of lipide metabolism in man has been the lack of adequate micromethods for the isolation and identification of the major lipide complexes present in the plasma and the individual fatty acids associated with these groups. In recent years, with the advent of new chromatographic procedures, two significant achievements have occurred in this sphere. By the use of silicic acid columns, Borgström (1, 2) in 1952 and Fillerup and Mead (3) in 1953 separated lipide extracts into sterol ester, triglyceride, free fatty acid and phospholipide fractions. Crombie, Comber, and Boatman (4) extended the reverse phase partition chromatographic technique of Howard and Martin (5), and while encountering considerable overlapping of zones, demonstrated the separation of milligram quantities of certain common saturated and unsaturated fatty acids contained in natural mixtures.

The present investigation was undertaken in an effort to combine and modify these methods in order to determine: (a) the nature and quantity of the specific higher saturated and unsaturated fatty acids that form ester linkages with the lipide complexes of the plasma of man and (b) the rates of synthesis of these acids following the administration of acetate-1-C^{14}.

METHODS AND MATERIALS

Part 1. The separation of the total lipide extract of the plasma into the total fatty acids of the sterol esters, the triglycerides and the phospholipides

a) *Extraction.* Four patients with limited life expectancies due to carcinoma of the lung without evidence of metastases were selected for study. All were considered to be in a good nutritional state and clinically free from gross metabolic disturbances. The subjects were maintained on normal hospital diets during the course of the investigation. Two hundred microcuries of acetate-1-C^{14} (specific activity 1.0 mc. per mM) were dissolved in a convenient volume of tap water and administered orally to all patients in the postabsorptive state on the morning of the experiment. Serial blood samples of approximately 60 ml. were taken in heparinized syringes beginning one hour after the administration of acetate and continued at intervals for 96 hours. Thirty-five ml. of plasma was then removed after centrifugation and rapidly blown into a one-liter Erlenmeyer flask containing 525 ml. of a 4:1 mixture of dimethoxymethane: methanol (6). At this point in some preliminary experiments, individual carbon-14 or tritium labelled lipides of known specific activity [2] were dissolved in one ml. of petroleum ether and added to the flask. The mixture was allowed to boil momentarily by cautiously rotating the flask under a hot water tap. After cooling for fifteen minutes, with frequent swirling, the mixture was filtered through an 18.5 cm. Whatman No. 1 filter paper into a one-liter round bottom flask with a side arm. The precipitate was washed twice with an additional 30 ml. of the dimethoxymethane: methanol mixture and the washings added to the lipide extract contained in the flask. The flask was placed on a constant temperature water bath maintained at 50° C and the clear yellow solution was concentrated to a small volume under reduced pressure. Throughout this procedure oxygen-free nitrogen was allowed to flow onto the surface of the liquid via the side arm. The capillary tip by which the nitrogen was delivered was not placed below the surface of the liquid since this tended to induce excessive foaming as evaporation proceeded. Forty ml. of warmed

[1] This work was supported by a grant (AT(30-1)-1846) from the Atomic Energy Commission. Additional aid was obtained from institutional grants from the Fluid Research Fund of Yale University and the American Cancer Society.

[2] The labelled radiochemicals were obtained from the following sources: a) Triolein—courtesy of Dr. David Kritchevsky, American Cyanamid Co., Pearl River, N. Y. (7). b) Tripalmitin, tristearin, palmitic acid, and stearic acid—Isotopes Specialties Co., Burbank, Calif. c) Sterol Ester—prepared according to the procedure of Borgström (8). d) Phospholipides—prepared biosynthetically following the administration of acetate-1-C^{14} to a subject. The phospholipides of the lipide extract of the plasma were obtained by precipitation with cold acetone and ethanolic $MgCl_2$ followed by repeated washings.

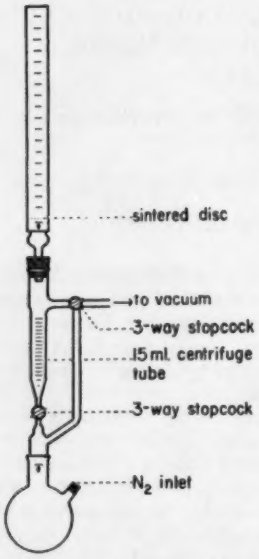

FIG. 1. SILICIC ACID CHROMATOGRAPHIC SYSTEM FOR THE SEPARATION OF THE MAJOR LIPIDE COMPLEXES OF THE PLASMA

Chromatographic tube 400 by 19 mm. The lower 3-way stopcock has an outlet to the atmosphere and is attached to a standard 24/40 ground glass joint.

petroleum ether (b.p. 30° to 60° C) was then added to the flask, the flask was stoppered with a ground glass stopper and shaken vigorously for two minutes. The supernatant petroleum ether layer was transferred to a 300-ml. round bottom flask with a side arm by means of a simple thin glass tube siphon device attached to water suction. The petroleum ether extraction was then repeated four more times. It was found that for quantitative recoveries, this technique of transfer was superior in all respects to that employing the use of separatory funnels.

The petroleum ether extracts were combined and the solvent was evaporated off under nitrogen at reduced pressure as described. The total lipide extract was then stored under nitrogen at −4° C until it was to be chromatographed.

b) *Chromatographic separation on silicic acid columns.* A snugly fitting piece of coarse filter paper was placed on top of the sintered disc at the bottom of the chromatography tube depicted in Figure 1. Twenty grams of silicic acid (100 mesh, Mallinckrodt) was thoroughly mixed with 4 grams of "Hyflo-Supercel" (Johns-Manville) and added to the tube. This ratio of filter aid to absorbent was found to provide excellent resolution with an adequate flow rate. The mixture was then packed by tapping the tube gently on a firm surface. Another filter paper disc was placed on top of the column and the column was washed with 70-ml. portions of absolute methanol, acetone, anhydrous peroxide-free ether and petroleum ether (3). The lipide extract to be chromatographed was dissolved in four to eight ml. of warmed 1 per cent ether in petroleum ether and quantitatively added dropwise to the top of the column. The sides of the column were then washed down with an additional five to ten ml. of solvent. In preliminary experiments (Table I), elution was carried out with the solvent systems recommended by Fillerup and Mead (3, 9). Since it was noted that some of the phospholipide fraction was eluted prematurely by solvent systems containing moderate concentrations of diethyl ether, the following technique was adopted to separate the sterol ester, triglyceride and phospholipide components of the plasma. Following the concentration of the total lipide extract prior to chromatographic separation, this material was taken up in a small volume of petroleum ether and quantitatively transferred to a heavy walled 40-ml. centrifuge tube. The final volume was reduced to about two to four ml. by evaporating off the excess of petroleum ether under a stream of nitrogen. Thirty ml. of cold acetone and ten drops of saturated $MgCl_2$ in ethanol were then added to the tube to precipitate the phospholipides. The tube was agitated and then placed in a refrigerator and allowed to stand overnight. Following centrifugation, the supernatant which contained the entire lipide extract with the exception of the phospholipides was siphoned off into a 200-ml. round bottom flask with a side arm. The precipitated phospholipides were washed two to three times with 30-ml. portions of cold acetone. After repeat centrifugation the acetone supernatants were then combined. Recoveries for phospholipides by this technique ranged from 97 to 102 per cent. Control cross contamination experiments performed by the addition of carbon-14 labelled sterol, fatty acid, triglyceride, and biosynthetically prepared radioactive phospholipide usually demonstrated less than 1 per cent contamination of the phospholipide precipitate by non-phospholipide constituents. Similar results were obtained when the acetone supernatants were assayed for contamination by the phospholipides. These findings are in accord with the observations of Borgström (1) employing the same technique.

The combined acetone supernatants were evaporated to dryness under nitrogen at reduced pressure. The residue was taken up in two to five ml. of warmed 1 per cent ether in petroleum ether and applied to the top of the silicic acid column as described. The sterol esters were eluted with 300 ml. of 1 per cent ether in petroleum ether; the triglycerides with 350 ml. of 3 per cent ether in petroleum ether. An elution rate of approximately 150 ml. per hour was maintained in all experiments by means of a "Manostat" attached to a vacuum pump. After separation the eluates were evaporated down to dryness as previously described, taken up in petroleum ether and then quantitatively transferred to a 100-ml. round bottom flask with a side arm. The phospholipide precipitate was also dissolved in a small volume of warm petroleum ether and transferred in a similar fashion.

c) *Saponification.* To each flask were added 20 ml.

TABLE I

Distribution of added radioactivity appearing in the various chromatographed fractions of plasma lipides in order of elution

Solvent system	Radioactive tracer*	Sterol esters 300 ml. 1% ether in pet. ether	Triglycerides 300 ml. 3% ether in pet. ether	Free fatty acids 225 ml. 10% ether in pet. ether	Sterol 225 ml. 50% ether in pet. ether	Combined fraction Free fatty acids+Sterol eluted with 300 ml. 50% ether in pet. ether	Phospholipides 225 ml. 25% methanol in ether	Recovery of radioactivity in specific fraction *per cent*
A	Triolein-H^3 3,120	19	2,995	79	18		3	96
	Tripalmitin-1-C^{14} 15,140	36	14,750	177	34		29	97
	Stearic acid-1-C^{14} 13,040	30	51	6,054	5,911		71	46
	Palmitic acid-1-C^{14} 11,017	37	43	5,105	5,417		84	46
	Cholesterol-4-C^{14} 20,980	24	97	14,070	5,890		228	28
	Cholesterol-4-C^{14} 20,797	36	106	13,895	6,057		185	24
B	Sterol Ester-C^{14}† 8,622	8,508	143			24	47	98
	Tripalmitin-1-C^{14} 13,955	52	13,987			98	26	100
	Stearic acid-1-C^{14} 11,737	26	412			11,521	158	98
	Cholesterol-4-C^{14} 16,336	56	145			15,636	238	95
	Phospholipide-C^{14}† 1,991	28	83			170	1,715	86

* Counts per minute added to the column.

† Typical examples of the separation of certain lipide fractions by chromatography with silicic acid using various solvent systems.

of 95 per cent ethanol and 0.7 ml. of 90 per cent aqueous KOH. A small Soxhlet water condenser was attached to the top of each flask, a stream of nitrogen introduced via the side arm, and the material refluxed for 90 minutes in a boiling water bath. During the procedure, evaporation of approximately 50 to 75 per cent of the ethanol occurred. The samples were then removed from the bath and allowed to cool. Five ml. of H_2O and twenty ml. of 95 per cent ethanol were added to each flask. The mixture was extracted three times with petroleum ether. The petroleum ether washings were combined and washed once with alcoholic KOH. This ethanol wash was added to the original aqueous material remaining in the flask and the petroleum ether extracts were then discarded. The mixture was made acid to phenophthalein with 6 N H_2SO_4. Ten to twenty ml. of H_2O were added and the mixture was then extracted three times with 20-ml. aliquots of petroleum ether. The combined petroleum ether extracts were washed once with 5 per cent sodium bicarbonate, three times with distilled water, and then dried over anhydrous sodium sulfate. The solvent was evaporated off under nitrogen at reduced pressure and the fatty acid residue was transferred to a 25-ml. volumetric flask and brought up to volume with petroleum ether.

d) *Assay.* Portions of these samples were then taken in duplicate, placed in small test tubes and the solvent blown off with a stream of nitrogen. The residue was taken up in 2 ml. of 65 per cent acetone in water (v/v) and two drops of bromthymol blue added. The total fatty acids of each class of lipides was then determined by titrating the sample under nitrogen against 0.005 N KOH in a microburette calibrated in 0.001 ml. In experiments where radioactive tracers were employed, another sample was removed from the volumetric flask, transferred to a 30-ml. counting vial (Kimble K-10 "Opticlear") and evaporated to dryness. To this vial was added 15 ml. of toluene containing 0.4 per cent of the phosphor, 2,5 diphenyloxazole and 0.005 per cent of the wave length shifter 1, 4-di-[2-(5-phenyloxazolyl)]-benzene (10). The radioactivity contained in the vial was determined in a dual channel liquid scintillation counter[3] which had an efficiency of 75 per cent for carbon-14 (with an 11:1 energy acceptance window and a background of 30 cpm.) and 22 per cent for tritium. All counts were corrected for background and counted to a standard error of 2 per cent.

Part II. The isolation of individual higher saturated and unsaturated fatty acids commonly found in ester linkage with the major lipide complexes

a) *Preparation of kieselguhr columns.* Five pounds of "Hyflo Supercel" (Johns-Manville) were thoroughly

[3] Technical Measurements Corporation, New Haven, Conn.

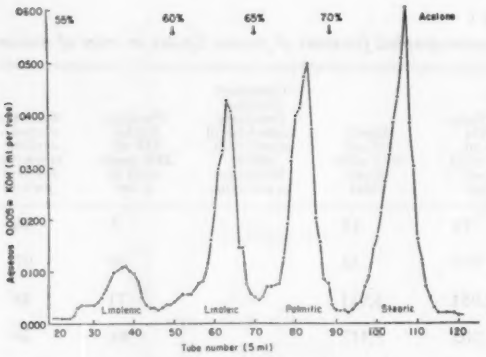

FIG. 2. THE RESOLUTION OF A MIXTURE OF LINOLENIC, LINOLEIC, PALMITIC, AND STEARIC ACIDS

The arrows represent the points where the aqueous acetone solvent systems were changed. Column, 130 by 1.0 cm.

mixed with several gallons of distilled water in a high wide-mouth glass container. The mixture was allowed to settle for ten minutes, after which time the fine particles that float were removed by aspiration. The washing process was repeated three to four more times. The Celite was then transferred to large glass trays, excess water decanted, and the tray placed in a drying oven maintained at 110° for 24 hours. The material should be thoroughly dry before further processing. The Celite was then transferred to large evaporating dishes and placed in a large vacuum desiccator at the bottom of which was 20 ml. of dichlorodimethylsilane in a wide-mouth container. The desiccator was partially evacuated and the silane vapors allowed to remain in contact with the Celite for four hours. The Celite was then removed and placed in a 3-liter beaker filled with water. The mixture was vigorously stirred and the particles that did not float were discarded. The coated Celite was transferred to a large Buchner funnel and washed several times with water and then methanol until the washings appeared neutral when tested with pH paper. The material was then thoroughly dried in an oven maintained at 80° C.

The most satisfactory separation of certain mixtures of the higher saturated and unsaturated fatty acids was obtained by using a jacketed column 1 cm. in diameter and 130 cm. in height equipped with a small reservoir at the top and an "Ultramax" valve at the bottom. For a column of this size, 48 grams of coated Celite and 34.8 grams of Standard White Oil No. 15 USP[4] were placed in separate beakers. One hundred ml. of anhydrous peroxide-free ether was added with stirring to the mineral oil. The Celite was then added. The beaker was placed in a warm bath and the mixture gently stirred to hasten the evaporation of the solvent. The coarse powder was then dried in a vacuum oven at 60° C for two hours. A convenient volume of 70 per cent acetone in water (v/v) was added and the mixture agitated for one minute in a Waring Blender to dissolve any lumps. To expel the air bubbles, the Celite was transferred to a beaker and warmed gently on a steam bath until bumping occurred. The slurry was then added to a chromatographic column filled with 70 per cent acetone by means of a large-bore dropping pipette. The Celite was uniformly packed by gravity by opening the column and allowing it to flow at a maximum rate. Air-free columns have been obtained by observing two precautions: a) the tip of the pipette by which the slurry is delivered should be placed below the meniscus of fluid at the top of the column before discharging the contents; b) the temperature of the water flowing through the jacket of the column should be the same as that of the slurry and the solvent systems (room temperature). A filter paper disc was placed on top of the Celite column and the column was then washed with 150 ml. of 55 per cent acetone before use.

b) *The loading of fatty acids on the column* (*11*). Ten to twenty-ml. samples of the total fatty acids of various lipide classes that were dissolved in petroleum ether and placed in volumetric flasks were removed and placed in a 50-ml. beaker. The solvent was evaporated off on a warm water bath under a stream of nitrogen. The residual fatty acids were then dissolved in 0.4 ml. of Standard White Oil and 15 ml. of anhydrous peroxide-free ether. Then 0.52 gram of coated Celite was added. The beaker was placed in a tray of warm water, and with constant stirring under a stream of nitrogen, the ether was blown off. The thoroughly dried powdery mull was then added to the top of the chromatographic column with a spatula. The beaker was rinsed several times with several ml. of warmed 55 per cent acetone and the washings were also added to the top of the column. The mull containing the acids was gently agitated with a glass rod and allowed to settle by gravity. Another filter paper disc was then placed on top of the mull.

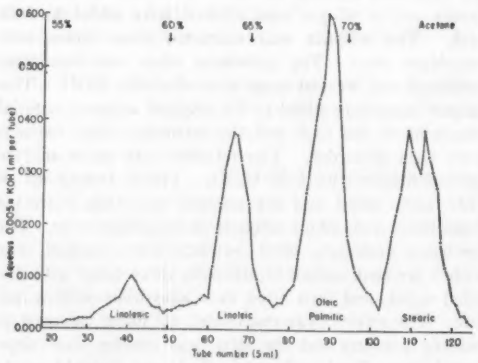

FIG. 3. RESOLUTION OF A MIXTURE OF LINOLENIC, LINOLEIC, PALMITIC, OLEIC, AND STEARIC ACIDS

Note that palmitic and oleic acids come off the column together.

[4] Standard Oil Company of California.

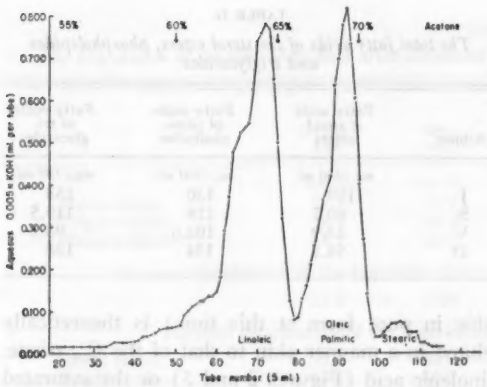

FIG. 4. THE CHROMATOGRAPHIC SEPARATION OF THE TOTAL FATTY ACIDS OF STEROL ESTERS OF THE PLASMA OF SUBJECT J

The solvent systems employed in sequence in the separation of known mixtures or plasma extracts containing the common higher fatty acids were as follows:

55 per cent acetone elutes arachidonic acid
linolenic acid
lauric acid
60 per cent acetone elutes linoleic acid
myristic acid
65 per cent acetone elutes palmitic acid
oleic acid [5]
70 per cent acetone elutes stearic acid

The systems were changed at intervals noted in Figures 2 to 6. The capacity of the column for total fatty acids was approximately 60 milligrams. The columns may be used repeatedly (5 to 10 times) provided they are kept at a constant temperature. Under these conditions, elution was quantitative and the removal of individual fatty acids with predetermined volumes of the various solvent systems could be anticipated.

c) *Collection and assay of samples.* The columns were mounted on an automatic fraction collector adjusted to collect 5-ml. samples every three minutes. The samples were titrated under nitrogen with 0.005 N KOH to a blue-green endpoint using bromthymol blue as an indicator. The contents of the tubes representing individual fatty acids were pooled, made acid with 6 N H_2SO_4, and concentrated under nitrogen at reduced pressure. The fatty acids were then extracted with petroleum ether, washed, dried and assayed for content and radioactivity as previously described.

[5] For weight measurements this fraction was subjected to the Bertram oxidation procedure (4) and then rechromatographed for the quantitative estimation of palmitic acid. The amount of oleic acid present in the original mixture was then obtained by subtracting the weight (by titration) of the recovered palmitic acid from that of the acids eluted by 65 per cent acetone during initial chromatography.

RESULTS

Nature and quantity of the fatty acids found in ester linkage with the various lipide complexes of the plasma

Typical examples of the resolving power of the silicic acid columns for the separation of the sterol esters and the triglycerides of the plasma using the solvent system recommended by Fillerup and Mead (3) are shown in Table I. In a series of quantitative experiments, recoveries of 95 per cent or more were constantly achieved for these fractions. Attempts at further fractionation of the lipide extract by this technique, however, were generally unsuccessful. Considerable cross contamination between "free fatty acids" [6] and sterols was noted. The gross overlapping of zones that occurred could not be obviated by either increasing the column length or altering the concentrations of the solvent systems employed in this investigation. Although the phospholipide fractions were generally eluted in a pure state by 25 per cent methanol in ether, the yields were frequently 10 to 20 per cent lower than those obtained by precipitation of the phospholipides in the cold by acetone and $MgCl_2$. Losses appeared to be due primarily to the premature separation of small quantities of

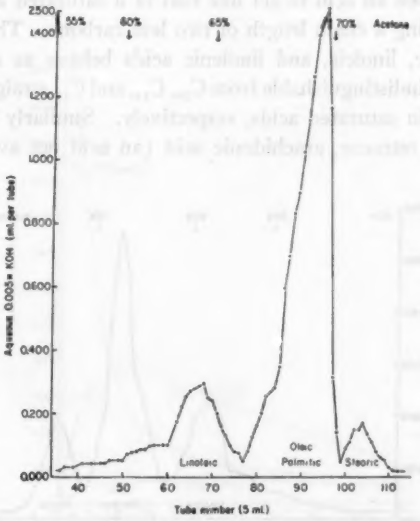

FIG. 5. THE CHROMATOGRAPHIC SEPARATION OF THE TOTAL FATTY ACIDS OF THE TRIGLYCERIDES OF THE PLASMA OF SUBJECT J

[6] Added radioactive fatty acids.

phospholipide when higher concentrations of diethyl ether were used in the elution of "free fatty acids" and sterols from the column.

The quantities of total fatty acids found in ester linkage with each of the major lipide complexes of the plasma are noted in Table II. These values compare favorably with those obtained by indirect measurements (12). The fatty acids of the triglycerides in the post-prandial plasma were usually present in the highest concentration, followed by the fatty acids of the phospholipides and then those of the sterol esters.

The results of the qualitative and quantitative resolution of known mixtures of some of the common straight-chain higher saturated and unsaturated fatty acids are depicted in Figures 2 and 3. Highly satisfactory separation of linolenic, linoleic, palmitic or oleic and stearic acids was accomplished by using kieselguhr column 1 cm. in diameter and 120 cm. in height. Preliminary experiments with shorter columns led to adequate resolution of palmitic and stearic acids. However, considerable overlapping was noted in the zones distinguishing linolenic from linoleic acid and linoleic from palmitic or oleic acid. As noted by previous investigators (4), the presence of each double bond some distance from the carboxyl group caused an acid to act like that of a saturated acid having a chain length of two less carbons. Thus, oleic, linoleic, and linolenic acids behave as and are indistinguishable from C_{16}, C_{14}, and C_{12} straight-chain saturated acids, respectively. Similarly the C_{20} tetraene, arachidonic acid (an acid not available in pure form at this time) is theoretically eluted in a manner akin to that of the C_{18} triene, linolenic acid (Figures 2 and 3) or the saturated C_{12} lauric acid.

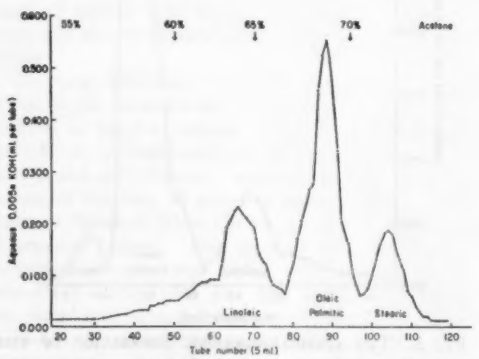

FIG. 6. THE CHROMATOGRAPHIC SEPARATION OF THE TOTAL FATTY ACIDS OF THE PHOSPHOLIPIDES OF THE PLASMA OF SUBJECT J

TABLE II

The total fatty acids of the sterol esters, phospholipides and triglycerides

Subject	Fatty acids of sterol esters	Fatty acids of phospholipides	Fatty acids of triglycerides
	mg./100 ml.	*mg./100 ml.*	*mg./100 ml.*
J	109	130	158
S	90.7	118	119.5
V	73.9	102.6	98.1
D	94.1	134	138

The chromatographic separation of the fatty acids of the sterol esters, the triglycerides, and the phospholipides of the plasma is depicted in Figures 4, 5, and 6. It is noted that the predominant components of all fractions are presumably linoleic, oleic, palmitic, and stearic acids. The various concentrations of the individual fatty acids associated with the major lipide complexes are listed in Table III. It should be stated that some titratable acidity, approximately two to four times that of the blank, occurred in the fractions eluted by 55 per cent acetone in water (v/v). Although this may represent very small quantities of either lauric (13), linolenic and arachidonic acids (4), or the products of partial oxidation of some of the higher unsaturated acids, no definite "hill and valley" elution pattern was noted. The very small band that did form tended to spread widely. This was probably due to the high water content of the solvent system employed under these circumstances (4, 5). Since the small quantities of these acids that were recovered precluded further accurate chemical identification at this time, they have been excluded from the table. It is, therefore, apparent that the small amounts of the higher unsaturated acids of the plasma, as determined by the alkali isomerization technique (14), are not easily discernible by this method at this time.

From an analysis of Table III, it appears that oleic and palmitic acids, respectively, represent the major unsaturated and saturated fatty acids of the plasma of man. It is also noted that the fatty acids associated with the sterol esters are predominantly unsaturated—linoleic and oleic acids constituting approximately 86 per cent of the titratable acidity

TABLE III

The distribution of the individual fatty acids associated with the major lipide complexes in the plasma of subject J *

	Linoleic acid	Oleic acid	Stearic acid	Palmitic acid	Total fatty acids of major complex	Per cent recovery of total fatty acids added to the column
	mg. %	*mg. %*	*mg. %*	*mg. %*	*mg. %*	
Sterol esters	57.7	31.9	2.2	11.5	103.3	
Triglycerides	18.9	68.9	7.3	45.1	140.2	89
Phospholipides	43.1	31.2	16.1	23.1	113.5	
Per cent distribution of the individual fatty acids recovered from the total fatty acid extract						
	33.5	37.0	7.2	22.3		
Per cent distribution of the individual fatty acids present in each major complex						
Sterol esters	55.8	30.9	2.2	11.1		
Triglycerides	13.4	49.2	5.2	32.2		
Phospholipides	37.9	27.5	14.1	20.5		

* Obtained by analysis of the chromatographically separated fractions.

recovered from the kieselguhr column after the addition of this fraction. Similarly, 65 per cent of the titratable acidity associated with the phospholipides and 62 per cent of that found with the triglycerides were attributable to these unsaturated acids. Palmitic and stearic acids, on the other hand, were more widely distributed in the triglyceride and phospholipide complexes, with only small quantities noted in the sterol esters.

It is highly probable that both the composition and concentration of the individual fatty acids associated with each of the major lipide complexes vary considerably with the diet (15–17).

Incorporation of acetate-1-C^{14} into the total fatty acids of triglycerides, the sterol esters and the phospholipides (Figure 7, Table IV)

The appearance of radioactivity in the total fatty acids of the triglycerides following the administration of the C^{14}-labelled two-carbon fragment was extremely rapid in all subjects. The specific activity of these substances invariably reached a maximum at two hours, declined sharply within a 24-hour period and then slowly declined during the next 72 hours. A semilogarithmic plot of the data demonstrates that decay does not occur by a single exponential process, and if additional points were taken over several more days the curves describing the decline of radioactivity probably could be resolved into a series of exponential rates. Thus, the calculation of a composite half-life for these fatty acids would not be very meaningful at this time. Undoubtedly a number of metabolic processes contribute to the disappearance of these materials from the blood stream (18).

The curve describing the specific activity of the fatty acids of the phospholipides increased more gradually to reach a plateau between 12 and 24 hours, at which point it intersected the specific activity curve of the fatty acids of the triglycerides and then fell off more slowly within the next 72

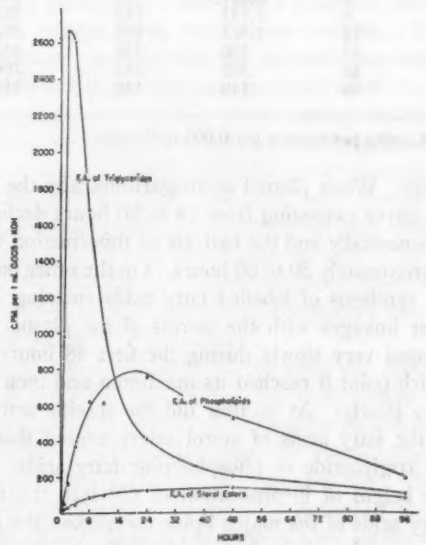

FIG. 7. THE INCORPORATION OF ACETATE-1-C^{14} INTO THE TOTAL FATTY ACIDS OF THE TRIGLYCERIDES, THE PHOSPHOLIPIDES, AND THE STEROL ESTERS OF THE PLASMA OF SUBJECT J

TABLE IV

Specific activity of the total fatty acids of the triglycerides, the sterol esters, and the phospholipides in the plasma

Subject	Time in hours	Fatty acids of triglycerides	Fatty acids of sterol esters	Fatty acids of phospholipides
		*CPM/ 0.005 mM**	*CPM/ 0.005 mM*	*CPM/ 0.005 mM*
J	1	984	20	61
	2	2,660	29	170
	4	2,600	48	378
	8	1,680	80	628
	12	1,050	96	320
	24	406	120	766
	48	202	126	532
	96	106	81	202
V	1	1,840	25	100
	2	5,705	41	251
	4	4,631	74	561
	8	3,689	110	933
	12	1,905	122	960
	24	729	151	1,280
	48	387	178	770
	96	187	103	320
S	1	2,025	10	51
	2	8,381	16	130
	4	5,267	25	270
	8	3,761	42	473
	12	2,470	45	627
	24	1,386	63	472
	48	540	69	395
	96	190	40	165
D	1	1,300	27	30
	2	3,625	60	80
	4	3,481	90	146
	8	2,733	152	322
	12	1,375	191	397
	24	560	226	332
	48	302	252	260
	96	140	140	110

* Counts per minute per 0.005 millimole.

hours. When plotted semilogarithmically the decay curve extending from 24 to 96 hours declined exponentially and the half-life of this fraction was approximately 50 to 60 hours. On the other hand, the synthesis of labelled fatty acids entering into ester linkages with the sterols of the plasma increased very slowly during the first 48 hours, at which point it reached its maximum and then fell very slowly. At no time did the specific activity of the fatty acids of sterol esters exceed that of the triglyceride or phospholipide fatty acids. At the height of incorporation of C^{14} into the total fatty acids of the major lipide complexes, the specific activity of the fatty acids of the triglycerides was two to four times that of the phospholipides and more than twenty times that of the fatty acids of sterol esters.

Incorporation of acetate-1-C^{14} into certain of the chromatographically separated fatty acids of the triglycerides, phospholipides and sterol esters (Table V)

The incorporation of radioactivity into certain of the individual fatty acids of the various lipide complexes followed more or less the same general pattern noted in the formation of the total fatty acids of each group. Thus, the specific activity of the palmitic, stearic, and oleic acids found in ester linkage with the triglycerides was maximum two to four hours after the administration of the acetate. Despite the difference in concentrations of these fatty acids in the plasma, palmitic acid showed the greatest specific activity at its peak, with less activity in stearic, and still less in oleic acid.

The presence of radioactivity in the fraction eluted by 60 per cent aqueous acetone (4) is probably due to contamination of the linoleic acid by the

TABLE V

Specific activity of the chromatographically isolated fatty acids of the triglycerides, the sterol esters, and the phospholipides in the plasma of subject J*

	Time in hours	Palmitic acid	Stearic acid	Oleic acid	Linoleic† and Myristic acid
Triglycerides	1	1,953	618	294	474
	2	5,409	2,433	1,497	906
	4	5,115	3,024	1,263	627
	8	—	—	—	—
	12	2,910	1,929	267	222
	24	663	552	252	105
	48	402	243	96	51
	96	171	96	81	—
Sterol Esters	1	96		30	NSC‡
	2	120		45	
	4	171		72	
	8	282		105	
	12	375		156	
	24	—		—	—
	48	495		201	
	96	351		120	
Phospholipides	1	183	72	63	
	2	432	210	159	
	4	942	465	297	
	8	1,563	723	534	
	12	1,578	672	555	
	24	1,890	1,110	825	
	48	1,287	804	414	
	96	—	—	—	

* Counts per minute per 0.005 millimole.

† Radioactivity in this fraction is presumably due to presence of small quantity of myristic acid (see Results).

‡ No significant counts above background.

very small quantity of myristic acid thought to be present in the plasma of man (13), which is also eluted from the chromatographic column by this solvent system. If this be the case, the myristic acid fraction would undoubtedly be diluted by the presence of presumably unlabelled linoleic acid (19–22). The specific activity of the myristic acid would therefore be greater than that stated, since the total acidity of the combined fraction was used in the calculation. This fraction was not subjected to the Bertram oxidation procedure (4) for the separation of the saturated from unsaturated acids because of difficulties encountered in handling very small quantities of saturated acids by this method, as would be the case with myristic acid in this particular instance.

The curves describing the appearance of C^{14} in the palmitic, stearic, and oleic acids attached to the phospholipides closely resemble those of the total fatty acids of this fraction. The maximum specific activity was again attained approximately 24 hours after the introduction of the precursor. The relative distribution of counts within the individual fatty acids was somewhat similar to that noted in the triglyceride fractions. No significant radioactivity was found, however, in that fraction containing linoleic acid and possibly traces of myristic acid.

The relatively small amount of radiactivity noted in the fatty acids of the sterol esters was predominantly due to the presence of labelled palmitic and oleic acids. Although some labelling occurred, the quantities of stearic acid isolated from this complex were too small for accurate analyses for specific activity. Again there was no discernible radioactivity in the relatively large quantity of linoleic acid that was isolated.

DISCUSSION

It is known that the fatty acids entering into ester linkage with each of the specific major lipide complexes are mixtures which may vary in quantity and composition. In contrast to the triglycerides, the fatty acids of the cholesterol esters under normal circumstances contain large quantities of linoleic acid. It has been shown that this polyunsaturated acid is not readily synthesized by the mammalian organism (19–22) and is presumably derived *in toto* from the diet. The specific activity of the saturated and monoethenoid fatty acids of the "non-phospholipide" fraction in this and other studies (16, 18, 23, 24) would tend to be greater if the contribution derived from the large unlabelled linoleic acid fraction of the sterol esters and from the somewhat smaller fraction from the triglycerides was excluded from the calculations. Similarly, other polyunsaturated fatty acids such as linolenic and arachidonic acids thought to reside in the phospholipide and sterol ester complexes (25) would also tend to alter the estimation of the specific activity of these fractions. The degree to which this occurs, however, would depend upon certain characteristics of some of the polyethenoid acids. Although relatively little is known about the intermediary metabolism of the higher unsaturated fatty acids, it has been shown in animals that some of the processes leading to the formation of one essential fatty acid from another may involve endogenous synthesis. Thus, Widmer and Holman (21) noted that fat-starved rats apparently synthesized arachidonic acid from linoleic, but not from linolenic acid. Further investigation of the problem by Mead, Steinberg, Howton, and Slaton (20, 22) demonstrated the formation of arachidonic acid from an exogenous precursor presumably derived from linoleic acid by the addition of a two-carbon fragment obtained from administered radioactive acetate. It is, therefore, apparent that the variation between the actual and theoretical specific activities of the total fatty acids, particularly of the phospholipide and sterol ester fractions, would also be dependent upon the extent to which labelled acetyl coenzyme A is utilized to form tetraenes and conceivably other highly unsaturated acids that may exist in small quantities in the plasma of man. Because of difficulties inherent in the isolation of these acids in the pure state, the specific activities listed in this study are only relative.

The curves (Figure 7) depicting the synthesis of the labelled fatty acids of the triglycerides (A) and the phospholipides (B) resemble that of a time course curve demonstrating the relationship between a precursor (A) and product (B) when a labelled precursor (acetate) is furnished at zero time (24). Since it has been implied on the basis of experiments in animals (23, 26) as well as man (18) that the triglyceride fraction is apparently the major vehicle for the transport of fatty acids in

the plasma, it seems likely that at least certain of the individual fatty acids of the phospholipides such as palmitic, stearic, and oleic acids are derived in part from or are exchanging with the fatty acids of the triglycerides. The same reasoning may apply to a limited extent to the fatty acids of the sterol esters. However, the lack of adequate studies concerning the relative rates of synthesis of this fraction in the mammalian liver and its presence in other tissues precludes further speculation on this point.

Some information concerning the interconversion of fatty acids may be derived from an analysis of the relative specific activities of the individual acids. It may be noted that despite the different concentrations of these substances in the plasma, the turnover rates of palmitic, stearic, and oleic acids within a major lipide complex are approximately comparable. Furthermore, the greatest degree of radioactivity was found in the palmitic acid fraction of each group, followed then by stearic and oleic acids. It would appear, from the work of Dauben, Hoerger, and Peterson (27), that palmitic acid for the most part is synthesized directly from two-carbon units and the amount derived from the process of elongation of an intermediate fatty acid such as myristic by the addition of a two-carbon fragment is exceedingly small. On the other hand, Stetten and Schoenheimer (28) and Zabin (29) have indicated that a significant quantity of stearic acid is formed by direct elongation of the carbon chain of palmitic acid by two-carbon atoms. Furthermore, the formation of the monounsaturated oleic acid probably occurs in a manner similar to that of the saturated acids. Indeed, Anker (30) studied the relative distribution of the isotope in the various higher fatty acids after feeding myristic acid-1-C^{14} to rats and concluded that the 14 carbon atoms of myristic acid were utilized for carbon atoms 5 to 18 of oleic acid by way of palmitic and stearic acids. A similar investigation employing acetate-1-C^{14} by Dauben, Hoerger, and Peterson (27) gave support to this postulate by showing that the pattern of distribution of the isotope in the degraded unsaturated C_{18} acids was identical with that of the saturated acids. Additional information on the metabolic interrelationships of these acids was provided by Weinman, Chaikoff, Dauben, Gee, and Entenman (31) who found that when palmitic acid was catabolized *in vivo*, it was primarily converted to small carbon units without any appreciable formation of acids of intermediary carbon length. In contrast, the catabolism of stearic acid (32), while similarly breaking down to short chain units, also gave rise to an appreciable quantity of palmitic acid. The relative concentration of C^{14} in the palmitic, stearic, and oleic acid fractions isolated in this study would tend to support the occurrence of these overall reactions in man. Thus, it can be assumed that palmitic acid is the major higher saturated fatty acid intermediate formed from acetate. At least three pathways seem to be involved in the further metabolism of this acid. First, some palmitic acid is undoubtedly deposited in the fat depots as such. Second, a quantity is utilized as fuel by breaking down to two-carbon fragments which can enter the tricarboxylic acid cycle and provide a source of energy. Lastly, a portion of the palmitic acid pool is converted to stearic acid. Only small quantities of this C_{18} acid are found in the plasma of man under normal circumstances. Presumably much of this acid is either oxidized for energy, desaturated to form oleic acid or reconverted to palmitic acid.

The absence of appreciable radioactivity in the linoleic acid fraction of the major lipide complexes of the plasma lends support to the contention that this diene cannot be detectably synthesized by the mammalian liver (19, 20, 22, 28). However, it has been shown recently by Mead, Slaton, and Decker (33) that at least the carboxyl carbon of linoleic acid can be utilized to a limited extent in the formation of some of the higher saturated fatty acids in the rat. The metabolic pathway involved in this reaction is not clear at this time. It may be mediated via a higher unsaturated acid such as arachidonic which can be formed from a linoleate derivative (20, 22) by the addition of a two-carbon fragment or by the direct conversion of linoleic acid to short chain or more intermediate units which then can be partially utilized in the synthesis of the saturated acids. This latter concept seems to be the more likely possibility.

SUMMARY

Certain specific saturated and unsaturated fatty acids associated with the major lipide complexes

of the plasma of man were isolated and measured by chromatographic methods. The predominant fatty acid components of the sterol esters, the phospholipides, and the triglycerides were linoleic, oleic, palmitic, and stearic acids. The largest quantity of unsaturated acids was noted in the sterol ester fraction. The majority of the saturated fatty acids were present in the triglycerides. The rates of synthesis of the total and certain of these individual higher fatty acids of man following the administration of acetate-1-C^{14} were also studied. Some of the fatty acids of the phospholipides appeared to be derived in part from those of the triglycerides. The triglycerides probably represent the major vehicle for the transport of fatty acids in man as well as animals. The highest concentrations of radioactivity appeared in the palmitic acid fraction of each of the major lipide complexes, followed by those in the stearic and oleic acids. The absence of demonstrable activity in the isolated linoleic fractions signified the lack of endogenous formation of this polyunsaturated fatty acid in man.

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RAPID AND SLOW COMPONENTS OF THE CIRCULATION IN THE HUMAN FOREARM [1]

By EDWARD D. FREIS, HAROLD W. SCHNAPER, AND LAWRENCE S. LILIENFIELD [2]

(From the Cardiovascular Research Laboratory, Georgetown University School of Medicine and the Veterans Administration Hospital, Washington, D. C.)

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The present study is concerned with the manner in which a segment of arterial blood flows through the vessels of the human forearm. Specifically it is directed toward answering the question whether blood flows as a unit or is distributed into channels with varying rates of flow. The experimental procedure employed has as its basis the fact that the forearm circulation is small in comparison to that of the total body.

Volume dilution methods as commonly employed for measuring either cardiac output or total blood volume require a dose of dye or other labelling material sufficiently large to produce significant concentrations in the general circulation. In the cardiac output method only the upslope, peak and first portion of the downslope may be obtained before distortion occurs due to contamination with recirculating labelled material.

In the forearm, however, only a small quantity of dye or other labelling substances injected into the brachial artery produces easily measurable concentrations in the effluent veins. The portion of this small dose of dye which escapes into the general circulation becomes so well diluted that its concentration in vessels elsewhere in the body cannot be detected by ordinary spectrophotometric methods. Hence, the washout of the dyed segment of forearm arterial blood can be studied without contamination or distortion by significant amounts of recirculating injectate.

The purpose of this investigation was not to measure absolute blood flow of the forearm and hand. The validity of indicator-dilution methods has not been established for making such measurement in a peripheral area which has multiple venous drainage (1). Rather, the concern was with the characteristics of the uncontaminated downslope of the labelled blood in the area drained by the particular effluent vein being sampled.

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[2] Helen H. Millenson Memorial Fellow, Metropolitan Heart Guild, Washington, D. C.

MATERIALS AND METHODS

The subjects either were normal or patients on the hospital wards. They were all young or early middle-aged males. All patients were afebrile and ambulatory for at least one week prior to serving as subjects for these experiments. None were suffering from diseases of the cardiovascular system.

The method was similar to that described in a previous communication (2) except that the amount of dye injected was smaller. One-fourth ml. of a 0.5 per cent solution of the blue dye T-1824 was diluted in a mixture containing 3.5 ml. of deuterium oxide in saline and 0.2 ml. of 5 per cent sodium thiocyanate in distilled water. Three ml. of this mixture was injected into the brachial artery through a 20 gauge needle attached to a three-way stopcock. Thiocyanate and deuterium oxide were added to the mixture in order to study the time-concentration curves of these permeable substances, as will be described in future reports.

Immediately following the injection sampling was begun through a 17 gauge "thin-walled" needle threaded well into a large antecubital vein. Whenever possible a vein was chosen which appeared to drain the deep as well as the superficial structures. Samples were collected in the manner described previously (2), usually at intervals of 2 seconds, then 20-second intervals to 4 minutes, then 5, 6, 8, 10, 12 and 15 minutes, respectively. A sample was withdrawn from a vein in the opposite arm 3 to 5 minutes after the injection in order to rule out the possibility of significant amounts of recirculating labelled material.

Several precautions were necessary to insure valid results. The needle in the vein was directed against the stream of flow and the bevel so placed as to provide good outflow and adequate samples. Scanty samples resulted when the bevel was against the side of the vein, producing distorted curves probably due to delay and mixing in the needle and collecting catheter. In cases in which an adequate outflow could not be obtained (less than 0.5 ml. of blood per second) the results were discarded. The arterial needle also must be well placed, permitting unobstructed injection of the tracer materials,

and the injection must be accomplished evenly, gently and rather slowly (0.5 to 1.0 ml. per second) in order to avoid arterial spasm. Arterial spasm was recognized by a sudden temporary decrease in outflow from the venous drainage needle. When this occurred the results were discarded.

The dye concentrations in the plasma were determined spectrophotometrically using 1-ml. cuvettes. It usually was necessary to dilute the samples containing the peak dye concentrations (and the plasma blank) with saline in order to obtain accurate readings. Occasional hemolyzed samples either were discarded or were read after precipitating the proteins of the sample and plasma blank with acetone. The resulting dye density values were plotted on semi-log paper. Chromium^{51} labelled red cells were prepared and the radioactivity of the samples was determined by the method of Sterling and Gray (3).

Mean circulation time was calculated as follows: each concentration per unit time was multiplied by its respective time from the midpoint of injection. The sum of these products then was divided by the sum of the concentrations. The volume of the collecting system (needle, stopcock and collecting catheter) was measured and appropriate correction made for delay in the catheter.

RESULTS

Uncontaminated curves were obtained in 28 subjects, in 25 with the dye T-1824 and in 3 with Cr^{51} labelled red cells (Table I). A monophasic downslope was obtained in only 3 of these cases. In the remaining 22 patients receiving the dye and in the 3 cases receiving Cr^{51} labelled red cells the downslopes were biphasic. Representative biphasic curves are illustrated in Figure 1. After injection there was a brief, latent period following

TABLE I

Mean circulation times and ratios of flows and volumes in the forearm circulation

Case	Age	Diagnosis	Type of curve*	T	t_1	t_2	f_1/f_2	v_1/v_2
J. C.	27	U.R.I.	M	72				
C. F.	28	Toxic hepatitis, conval. 9 weeks	B + tr.	35	22	95	4.6	1.1
C. H.	32	U.R.I.	B − tr.	70	28	105	1.0	0.2
C. T.	30	Atypical pneumonia	B − tr.	79	56	283	5.1	1.4
H. F.	30	Lobar pneumonia	B + tr.	27	25	66	19.5	7.5
J. G.	33	Psychoneurosis	M	29				
G. P.	43	Peptic ulcer	B + tr.	125	50	178	0.7	0.2
J. O.	31	Diabetes mellitus	B + tr.	51	18	102	1.5	0.25
R. T.	31	Idiopathic pericarditis	B − tr.	61	55	210	26.0	6.8
A. G.	28	Early cirrhosis	B + tr.	63	21	128	1.5	0.25
H. W.	27	Psychoneurosis	B + tr.	38	22	140	6.4	1.0
M. W.	30	Peptic ulcer (Cephalic vein)	B + tr.	56	30	91	1.3	0.4
		(Median vein)	B + tr.	65	35	101	1.2	0.4
S. M.	48	Bronchial asthma	B − tr.	51	41	119	6.8	2.3
Hand only								
A. R.	31	U.R.I.	B + tr.	41	31	115	7.4	2.0
E. P.	40	Peptic ulcer	B + tr.	67	40	150	3.1	0.8
Forearm only								
J. G.	36	Erythema multiforme	B + tr.	35	23	139	8.7	1.4
E. C.	45	Bursitis	M	114				
J. H.	33	Peptic ulcer	B + tr.	102	49	239	2.6	0.5
W. C.	32	Urticaria	B − tr.	32	17	86	2.3	0.5
J. E.	38	No disease found	B − tr.	70	64	349	47.0	8.5
W. J.	27	Diabetes mellitus	B − tr.	110	21	176	0.7	0.1
A. M.	30	Psychoneurosis	B − tr.	90	35	136	0.8	0.2
J. M.	28	Latent syphilis	B − tr.	39	26	105	5.0	1.25
C. E.	24	No disease found	B + tr.	61	22	99	0.8	0.2
Cr^{51} RBC								
E. W.	34	Peptic ulcer	B − tr.	36	32	112	19.0	3.8
B. H.	54	Essential hypertension, mild	B + tr.	27	13	116	6.4	0.7
F. M.	48	Essential hypertension, mild	B + tr.	17	13	64	11.8	2.4
		Mean and S.D.		59.1 ±29.0	31.4 ±14.1	146.0 ±72.4	7.6 ±10.5	1.8 ±2.4
		Median					5.1	1.0

* M = Monophasic downslope; B + tr. = Biphasic downslope with transition zone; B − tr. = Biphasic downslope without transition zone.

which the dye appeared and rapidly increased in concentration forming a steep ascending phase lasting generally a few seconds. The curve fell away at first rapidly and then more slowly. The transition between the early and late phases of the downslope either was abrupt or connected by a short transition zone. The two phases of the downslope both were exponential forming straight lines when plotted on semi-log paper. In 10 cases the early steep downslope and later shallower downslope were connected directly without an intervening transition zone (Figure 1a). In the remaining 15 subjects a transition zone which was intermediate in gradient connected the early and late phases of the downslope (Figure 1b).

Although the majority of the curves showed clearly delineated biphasic downslopes there were some variations from the type of curves illustrated. The variations seen included irregularity of upslope, "sawtooth" or double peaks and small, late, third phase which was either shallower or steeper than the preceding phase. These variations usually were observed in the cases in which small samples were obtained presumably because of poor venous blood flow. In the calculations which follow, these variations were "rounded off" because they would be difficult to subject to mathematical treatment and were not representative of the majority of the curves.

The biphasic downslopes were not due to differences in velocity of blood flow through the hand as compared with the forearm. In 9 subjects the hand was excluded by inflating a cuff on the wrist to pressures 100 mm. Hg above systolic pressure (Table I). Typical biphasic downslopes were seen in 8 of these cases. In two subjects (A. R. and E. P., Table I) the injection was made into the radial artery and sampling was carried out through a vein in the wrist. Multiphasic downslopes were obtained in both instances.

Analysis of the biphasic downslope into components with different flows and volumes

Reasons are presented in the discussion for believing that the biphasic downslopes represent a rapid flow component producing the early steep downslope and a slower component producing the later shallow downslope. It was desirable to analyze these component parts quantitatively in terms

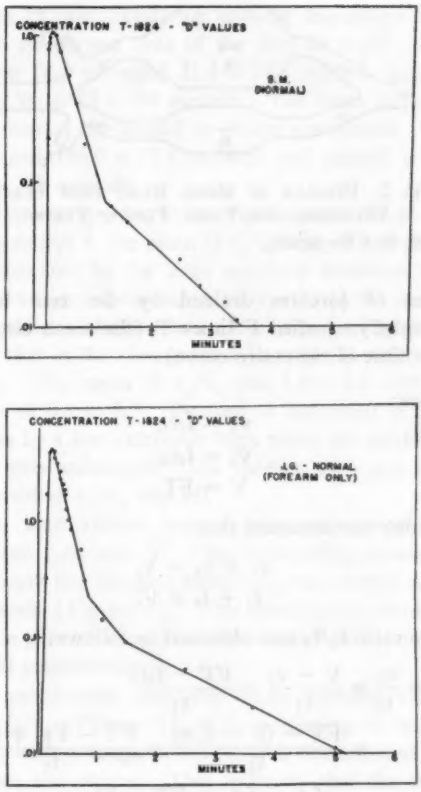

FIG. 1. GRAPHS ILLUSTRATING TYPICAL BIPHASIC TIME-CONCENTRATION WASHOUT SLOPES IN THE HUMAN FOREARM

Figure 1a (above) illustrates a simple biphasic exponential curve. Figure 1b (below) shows a biphasic curve with transition zone.

of flow and volume. Absolute measurements of flow were not possible because the dye is not always distributed uniformly in the forearm (1). However, the relative flows and relative volumes in the biphasic system could be determined using only the mean circulation times.

The solution was based on the well known relationship that vascular volume from the point of injection to the point of sampling is equal to blood flow multiplied by the mean circulation time. Thus, v_1, the vascular volume of the rapid component, was equal to f_1 (the flow in this component) times t_1 (its mean circulation). Similarly, v_2 (the volume of the slow component) equalled f_2 times t_2, and V (the total vascular volume of the seg-

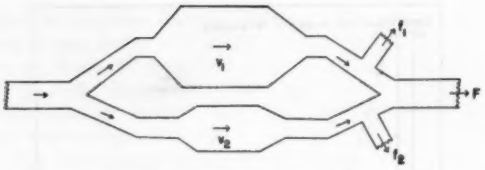

FIG. 2. DIAGRAM OF MODEL BUILT FROM PLASTIC CYLINDERS AND TYGON PLASTIC TUBING
See text for details.

ment of forearm drained by the vein being sampled) equalled F times T (the mean circulation time of the entire curve).

Thus:

$$v_1 = f_1 t_1,$$
$$v_2 = f_2 t_2,$$
$$V = FT.$$

It also was assumed that:

$$v_1 + v_2 = V,$$
$$f_1 + f_2 = F.$$

The ratio f_1/f_2 was obtained as follows:

$$f_1 = \frac{v_1}{t_1} = \frac{V - v_2}{t_1} = \frac{FT - f_2 t_2}{t_1}$$
$$= \frac{FT - (F - f_1)t_2}{t_1} = \frac{FT - Ft_2 + f_1 t_2}{t_1},$$
$$f_1 t_1 - f_1 t_2 = FT - Ft_2$$

or

$$f_1(t_1 - t_2) = F(T - t_2),$$
$$f_1 = \frac{F(T - t_2)}{t_1 - t_2}.$$

Similarly:

$$f_2 = \frac{F(T - t_1)}{t_2 - t_1},$$

$$\frac{f_1}{f_2} = \frac{t_2 - T}{T - t_1}, \quad \text{and} \quad \frac{v_1}{v_2} = \left(\frac{f_1}{f_2}\right)\left(\frac{t_1}{t_2}\right).$$

A simple model was prepared to test these assumptions (Figure 2). Plastic cylinders with gently sloping inflow and outflow tracts designed to minimize areas of stagnant flow and permit good mixing of the dye were designated v_1 and v_2. These were filled loosely with glass wool to further promote adequate mixing. These plastic vessels were connected with Tygon plastic tubing as shown in Figure 2 and the inlet was connected to a constant overflow reservoir. Tap water was permitted to flow through the system and T-1824 was injected into the common inflow tract.

By placing a resistance (screw clamp) in the inflow tract to either v_1 or v_2 the flows to either v_1 or v_2 could be adjusted at will. By clamping the common outflow tubing the flows through v_1 and v_2 could be measured separately either directly or by the dye dilution method. After several such determinations the side channels were clamped and the common outflow tubing opened. The dye again was injected in order to obtain the composite dye dilution curve.

One such experiment is illustrated in Figure 3. The solid lines represent the curves obtained from v_1 and v_2 separately. The broken line is the curve obtained when the two volume flows were permitted to mix and flow out of the common channel. After extrapolating the downslope of the first portion of the broken line curve and the upslope of the second portion t_1, t_2 and T, the respective mean circulation times, were measured, and from these the ratios of the flows and volumes. The ratio of flows indicated that f_1 was 6 times f_2 in comparison to the actual ratio of 8.5 times. The ratio of the volumes indicated that v_1 was twice v_2,

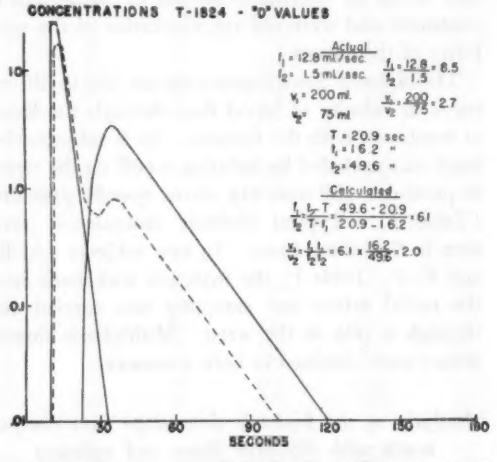

FIG. 3. CHART OF T-1824 TIME-CONCENTRATION CURVES OBTAINED BY PERMITTING TAP WATER TO FLOW THROUGH THE MODEL SYSTEM ILLUSTRATED IN FIGURE 2

The solid line curves were obtained by collecting the outflow from v_1 and v_2 separately. The broken line represents the curve obtained by collecting through the common outflow tubing. See text for details.

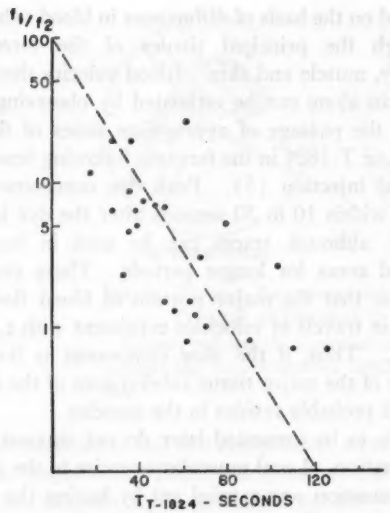

FIG. 4. GRAPH SHOWING THE RATIO f_1/f_2 PLOTTED ON LOG SCALE AGAINST LINEAR PLOT OF THE MEAN CIRCULATION TIME (T) OF THE ENTIRE CURVE

f_1/f_2 increases inversely to T.

which was a reasonable approximation of the actual value of 2.7 times.

It seemed probable that a closer approximation to actual values could have been obtained by taking greater pains to ensure adequate mixing and by making the system larger in order to prolong the collection period. However, we were satisfied that extrapolation of the composite biphasic curve gave a reasonable approximation of the two monophasic curves which represented the volume and flow in the constituent compartments.

Ratios of flows and volumes in the biphasic system of the forearm

The curves of the biphasic downslope were extrapolated by first projecting downward the straight line early phase of the downslope and then subtracting this extrapolated portion from the corresponding values of the second portion (or transition zone if present) of the actual downslope. The values obtained by such subtraction were plotted as the upslope of the second portion.

T, the mean circulation time of the entire curve, ranged between seventeen and 125 seconds (mean 59 ± 29 seconds) in the 28 subjects (Table I). In the 25 cases exhibiting biphasic downslopes the mean circulation time of the first or rapid component (t_1) averaged 31.4 ± 14.1 seconds with a range from 13 to 64 seconds. The mean circulation time of the second or slower component (t_2) averaged 146.0 ± 72.4 seconds and ranged from 64 to 349 seconds.

Flow was greater in the rapid component in all cases except 4, the mean of f_1/f_2 being 7.6 ± 10.5. As indicated by the large standard deviation the range was great, the values varying from 0.7 to 47. The volume of the rapid component was greater than that of the slow portion in only 11 of the 25 cases. The mean of v_1/v_2 was 1.8 ± 2.4 with a range of 0.1 to 8.5. Because of distortion of the means by a few extremely high ratios the medians also were calculated. The median f_1/f_2 was 5.1 and median v_1/v_2 was 1.0.

An approximate inverse relationship existed between f_1/f_2 and T. The relationship assumed a straight line function when f_1/f_2 was plotted on a log scale (Figure 4). This relationship implies that in general the greater the blood flow the more f_1 will predominate over f_2.

A relationship also was found between v_1/v_2 and f_1/f_2 (Figure 5), f_1/f_2 averaging 6 times v_1/v_2 with a range of 2.5 to 10.5 times including all but two values. This indicates that the volumes of the two components vary in such a way that as flow in the rapid component increases so does its relative volume.

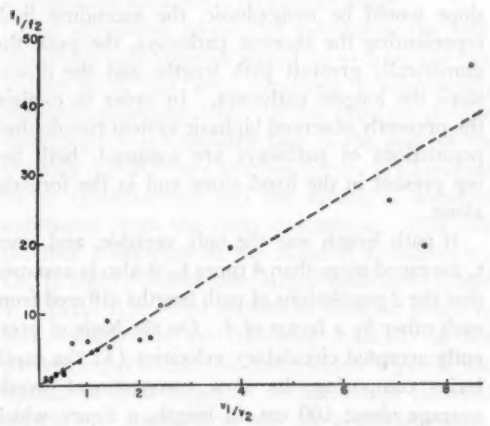

FIG. 5. GRAPH SHOWING THE RELATIONSHIP BETWEEN f_1/f_2 AND v_1/v_2

DISCUSSION

Several hypotheses can be advanced to explain the observed biphasic system. The first to be considered is differences in path lengths. According to Wiggers (4) the velocity of blood flow in the various subdivisions of the vascular tree varies inversely with the cross sectional area, decreasing gradually in the arteries from an aortic velocity of 18 cm. per second, slowing abruptly in the arterioles and capillaries where velocities of approximately 5 mm. per second exist, and increasing again in the large veins to 5 or more cm. per second. Assuming the longest probable path length in the forearm to be 80 cm. for both arterial and larger venous segments, 4 cm. for the arteriole, 2 cm. for the capillary, 4 cm. for venule and 10 cm. for the small venous segments, and employing conservative estimates of velocity in these segments, transit through the longest pathway would be less than 30 seconds. This fails to agree with the mean circulation times observed in the present experiments, since mean t_2 was 141 seconds or approximately 5 times the duration required for blood to flow through the longest pathway on the basis of presently assumed mean velocities (4).

The actual distribution of path lengths in the forearm would be expected to follow a typical population or "bell-shaped" distribution curve. If the velocities of flow were uniform in each subdivision throughout the forearm and difference in path lengths was the only variable, the downslope would be monophasic, the ascending limb representing the shortest pathways, the peak the numerically greatest path lengths and the downslope the longest pathways. In order to explain the presently observed biphasic system two distinct populations of pathways are assumed, both being present in the hand alone and in the forearm alone.

If path length was the only variable, and since t_2 averaged more than 4 times t_1, it also is assumed that the 2 populations of path lengths differed from each other by a factor of 4. On the basis of presently accepted circulatory velocities (4) the capillaries composing the slow compartment would average about 100 cm. in length, a figure which seems to be incredibly large.

The two compartment system also might be explained on the basis of differences in blood velocity through the principal tissues of the forearm, namely, muscle and skin. Blood velocity through the skin alone can be estimated by observing directly the passage of appropriate doses of fluorescein or T-1824 in the forearm following brachial arterial injection (5). Peak dye concentrations occur within 10 to 30 seconds after the dye is injected, although traces can be seen in heavily stained areas for longer periods. These studies indicate that the major portion of blood flow to the skin travels at velocities consistent with t_1 and not t_2. Thus, if the slow component is limited to one of the major tissue subdivisions of the forearm, it probably resides in the muscles.

Data to be presented later do not support this explanation. Local muscular exercise to the point of exhaustion was carried out by having the subject repeatedly squeeze a hand dynamometer. Dye curves following such local exercise revealed proportionate reductions of both t_1 and t_2 compared with the controls. Since muscle and not skin was affected predominantly by such exercise, and if muscle were the site of the slow component, there should have been a disproportionate reduction of t_2. In addition, in the untreated subject biphasic curves were observed in the hand alone, which contains little muscle, and in forearm alone (exclusive of the hand and wrist), which contains predominantly muscular tissue.

Renkin, utilizing a different technique, observed similar biphasic blood flow patterns in the perfused hindleg of the cat (6). The relative volumes of the two components were uninfluenced by removing the skin. The proportion of bone was too small to account by itself for one compartment and Renkin concluded that both compartments are present in skeletal muscle. These various observations make it appear unlikely that the two-phase velocity system is produced by differences in blood velocity in different tissues.

Barcroft presents evidence pointing toward a double circulation in the human forearm and calf (7). He believes that one is under neurogenic control, the other under the influence of local metabolites. The measurements were determined on mean blood flow and its response to various stimuli.

Whereas in the normal subjects flow in the rapid component usually predominated, the vas-

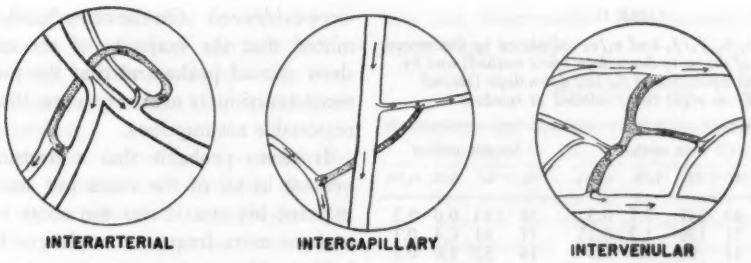

FIG. 6. TYPICAL ANASTOMOTIC CONNECTIONS BETWEEN ARTERIOLES, CAPILLARIES AND VENULES AS OBSERVED IN THE HAMSTER CHEEK POUCH PREPARATION

Arrows indicate the direction of flow. Arrows with heads pointing in opposite directions and stipled sections indicate anastomotic vessels with sluggish and reversible flow.

cular volumes of the two components were of the same magnitude. Thus, nearly half and in many of the subjects more than half of the vascular volume of the forearm contained slowly circulating blood. As has been outlined above the evidence pointed away from localizing rapid blood flow to one type of tissue and sluggish flow to another and suggested that the two flow systems existed side by side in both skin and muscle.

In seeking for the location of this large vascular component we have observed the circulation in the hamster cheek pouch according to the method of Lutz and Fulton (8). Using 100 to 300 times magnifications it was apparent immediately that the small vessel circulation has a biphasic pattern of blood velocity. In every field vessels of the same order were seen with widely varying rates of blood flow (Figure 6).

The mechanism for the variation of velocity was apparent. The circulation was highly anastomotic with many interarteriolar, intercapillary and intervenular connections. A similar anastomotic circulation has been described in the bat's wing (9, 10), in the subcutaneous tissue of mice (11), in voluntary muscle (12) and in intestinal wall (13). Since pressures at either end of the anastomosis were similar, flow was sluggish in the anastomosis. Indeed, it sometimes would stagnate, surge first one way and then the other and occasionally reverse.

Flow through this network of small vessels also was highly dynamic. A capillary or venule appearing to contain the rapid or main line flow would suddenly change into a sluggish channel. Similarly, slow moving flow in an anastomotic vessel would for short periods become rapid, moving sometimes in one direction, at other times in the opposite direction. Thus, not on'y do the rapid and slow moving circulations exist side by side but some of the small vessels change their role from time to time, now carrying rapid and at another time sluggish flow. In a system composed of anastomotic channels relatively minor shifts in pressure could easily change the pattern of blood flow. The factors controlling such pressure fluctuations are not completely understood although contraction of arterioles and precapillary sphincters probably play a part. Although these direct observations on the microcirculation of other preparations do not prove that the slow component of the forearm circulation in man is due entirely to retarded flow in anastomotic vessels, they provide a reasonable hypothesis for further study.

An alternative method of calculating the relative flows and volumes would be to treat the two curves as if they were occurring synchronously rather than successively. In this situation the late downslope is extrapolated back to the peak of the primary curve (since the two curves are assumed to be synchronous) and the values so obtained subtracted from the early downslope. This will produce a decrease in the ratios f_1/f_2 and v_1/v_2, *i.e.*, f_2 and v_2 will become larger relative to f_1 and v_1 (Table II). This method of analysis seems to lack validity for the following reasons: in the present experiments the observations began at the instant of first injection of the tracer material which was confined to the intravascular space. The tracer entered as a bolus and was preceded and followed by undyed blood. In essence, it

TABLE II

Comparison of t_1/t_2, f_1/f_2 and v_1/v_2 calculated by downward extrapolation of the early downslope (first method) and by backward extrapolation of the late downslope (second method) in eight cases selected at random

Case	T	First method				Second method			
		t_1	t_2	f_1/f_2	v_1/v_2	t_1	t_2	f_1/f_2	v_1/v_2
J. H.	102	49	247	2.7	0.5	38	143	0.6	0.2
A. G.	63	21	136	1.7	0.25	17	84	0.4	0.1
W. C.	32	17	86	2.3	0.5	14	52	1.0	0.3
C. F.	35	22	102	5.2	1.1	19	57	1.4	0.5
C. H.	70	28	105	1.0	0.2	23	77	0.2	0.1
A. R.	41	31	140	10.0	2.0	30	96	5.0	1.6
S. M.	51	41	119	6.8	2.3	38	80	2.3	1.1
E. P.	67	40	156	3.2	0.8	32	107	1.1	0.3
Mean	58	31	136	4.1	1.0	26	87	1.5	0.5

was a transient suddenly introduced into a flowing stream which advanced as a front.

If there are two radically different systems of dye transit time in the forearm it is obvious that the bolus will traverse the rapid component with greater speed than the slow component. As a result the appearance and peak times of the rapid component will precede those of the slow. Mixing will not be simultaneous in the two systems. Therefore, the assumption of synchronous rather than successive peaks does not seem to be applicable to this experimental situation.

At first glance it may appear that, since the two components probably are confined to the terminal arborizing network of small vessels, mixing will be simultaneous in both systems. This analysis is not justified, however. The traversal time of the dye is dependent upon the two factors of path length and velocity of flow. Reasons for believing that the biphasic pattern is due to differences in velocity rather than in path length have already been presented. Obviously the same small vessel cannot carry simultaneously both high and low velocity flow. Since the vessels must be separate at any given point in time, the analysis will not be affected whether they present an anastomotic network or two separate mixing chambers as in the model. Each chamber of the model also has to a lesser extent a population of path lengths (the central stream as compared to paths adjacent to the walls of the chamber) and of velocities (laminar flow).

Finally, it should be mentioned that in a few of the biphasic curves a second upslope and peak were evidenced. On the other hand, it must be admitted that the majority of the curves did not show second peaks and that the method of their reconstruction is arbitrary even though based on reasonable assumptions.

It seems probable that a transition zone was present in all of the cases but was not detected in some because it was too short to be apparent without more frequent sampling. The distinction between the presence or absence of a transition zone often was one of personal choice. For example, in Figure 1 it would have been possible to draw a transition zone in patient S. M.

When transition zones were delineated clearly it was found that extrapolation of the early downslope reached an insignificant concentration at a point on the abscissa which approximated the end of the transition zone or beginning of the late downslope. This would be expected if the transition zone represented the merging of the downslope of t_1 and the upslope of t_2. However, when the method of analysis dependent upon backward extrapolation of t_2 was used the resulting t_1 downslope reached insignificant concentrations at a point in time far short of the end of the transition zone. This would indicate that the contribution of dye from the rapid component had been dissipated at this point. This leaves no explanation for the transition zone except to postulate a relatively brief third component with no transition zones, which seems unlikely. Thus, backward extrapolation of the late downslope leads to serious difficulties in regard to the interpretation of the transition zone and provides an additional reason for discarding this method of analysis in the present experiments.

Regardless of the method of establishing the partition between "fast" and "slow" components the relationship between f_1/f_2 and T as well as v_1/v_2 and f_1/f_2 will be similar qualitatively as can be shown by plotting the representative cases listed in Table II. It should be emphasized that the terms "components" or "compartments" do not imply anatomically discrete systems. As will be shown in a later communication the ratios f_1/f_2 and v_1/v_2 change markedly in the same forearm under conditions of vasodilation, vasoconstriction or venous congestion. These changes in f_1/f_2 and v_1/v_2 continue to be related to T as in the present study. Similar to the hamster cheek

pouch circulation, therefore, some vessels may at one point in time be members of one component and at another point in time (and especially during major hemodynamic changes) become a part of the other component.

The present biphasic downslopes are not to be confused with the hyperbolic downslopes characteristic of dye curves obtained in a single tube containing laminar flow. The present curves were distinctly biphasic rather than hyperbolic. The "break" in the biphasic curve usually occurred after 60 seconds, which would be quite late if it were due only to the effects of laminar flow. Finally, labelled red cells which are not subjected to laminar flow also exhibited biphasic downslopes.

The question arises whether the biphasic pattern of blood flow is limited to a few body areas or is characteristic of the flow pattern in all organs. Data to be presented in a later communication indicate that the lungs and kidney of the dog exhibit biphasic downslopes although in these rapid flow areas the fast component predominates much more than in the forearm circulation.

SUMMARY AND CONCLUSIONS

Utilizing a method to prevent significant recirculation, the late downslopes of T-1824 or Cr^{51} labelled red cells were determined in the human forearm of 28 subjects. All but three of these curves exhibited biphasic, exponential downslopes.

Analysis of these slopes indicated that the forearm circulation can be divided into a rapid and a slow flowing component. In the normal subject the rapid component usually predominates, whereas the vascular volumes of the two compartments are nearly equal. With increasing forearm blood flow (as indicated by the mean circulation time) flow in the rapid compartment increases relative to that in the slower system. As the relative flow of the rapid component increases its relative volume becomes greater.

Evidence is presented to suggest that the site of the biphasic flow pattern is in the small vessel circulation. Sluggish flow is present in a high percentage of the many interarteriolar, intercapillary and intervenular anastomoses that characterize these vessels.

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THE GLUCOSE METABOLISM OF PATIENTS WITH MALIGNANT DISEASE AND OF NORMAL SUBJECTS AS STUDIED BY MEANS OF AN INTRAVENOUS GLUCOSE TOLERANCE TEST [1, 2]

By PAUL A. MARKS and JONATHAN S. BISHOP [3]

(From the Medical Services of The Francis Delafield Hospital and The Presbyterian Hospital, Department of Medicine, Columbia University College of Physicians and Surgeons, New York City, N. Y.)

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Since the observations of Freund (2) in 1885 of spontaneous hyperglycemia in patients with malignant disease, there has been speculation that an alteration in glucose metabolism is associated with neoplasia in man (3–18). There have been, however, relatively few studies designed specifically to test this possibility. Rohdenburg, Bernhard, and Krehbiel (5) and Edwards (6), with the advent of the oral glucose tolerance test, reported that a decrease in carbohydrate tolerance was a uniform finding in patients with cancer. Subsequent studies (7–18) did not confirm these reports, though several workers have found a high incidence of an abnormal glucose tolerance test in subjects with various types of neoplasms. These observations have been inconclusive with regard to establishing a relationship between the presence of uncomplicated neoplastic disease and an altered carbohydrate metabolism. A primary reason for the inconclusive nature of these studies is their lack of control of various factors known to decrease glucose tolerance. Certain of these factors, *e.g.*, inadequate dietary intake, fever, infection, hepatic dysfunction and prolonged bed rest, are frequently present in patients with malignant disease.

This study was undertaken in a group of carefully selected patients with chronic leukemia, lymphoma, and clinically early epithelial neoplasms, and subjects without cancer, in an effort to determine whether a defect in glucose metabolism is associated with malignant tumors. It was found that, as a group, the patients with neoplastic disease had a significant decrease in the rate of disappearance of glucose from the blood following the intravenous injection of glucose. Carbohydrate metabolism was evaluated further in these subjects by calculation of the net rate of disappearance of glucose from the blood and the volume of distribution of glucose and by determination of the changes in serum inorganic phosphate and potassium concentrations during the glucose tolerance tests.

METHODS

Subjects studied

The control group consisted of 19 individuals without neoplastic disease, 7 males and 12 females, ranging in age from 29 to 65 years. The neoplastic group included 36 individuals, 14 males and 22 females, ranging in age from 36 to 70 years. There was no significant difference between the control and neoplastic groups with respect to age or body weight. Subjects 20 through 33 had clinically localized carcinoma, which was in all instances considered amenable to curative therapy. These individuals had no systemic symptoms. Carcinoma was suspected on the basis of a finding of a lump in the breast on physical examination, a positive Papanicoloau smear on routine cervical examination, or an abnormal shadow on routine chest x-ray. Subjects 34 through 54 had lymphoma or chronic leukemia. The diagnosis in each individual was established histologically (Tables I and II). All subjects were studied preoperatively, or prior to chemotherapy or radiotherapy.

The criteria for selection of individuals for study in both the control and patient group included: 1) no family history of diabetes mellitus, 2) full ambulation, 3) no weight change for at least six months prior to the study, 4) no obesity, 5) no evidence or history of any disorder known to affect carbohydrate metabolism, 6) no fever, 7) normal serum concentrations of sodium, potassium, chloride, carbon dioxide, non-protein nitrogen, alkaline phosphatase, cholesterol and cholesterol ester, and a normal cephalin flocculation, and 8) an adequate dietary intake.

[1] A portion of the present data has been published previously in abstract form (1).

[2] This work was supported in part by a grant from The National Cancer Institute, U. S. Public Health Service.

[3] Fellow of the American Cancer Society.

TABLE I

Intravenous glucose tolerance test in control subjects

Subject No.*	Fasting glucose†	Glucose concentration (*mgm. %*) (*Time in minutes*) 8	16	24	32	40	48	56	64	Disappearance rate constant (*%/min.*) Total	Increment	Mean glucose concentration‡ (*mgm. %*) Total	Increment	Net glucose disappearance‡ (*mgm. %/min.*) Total	Increment
1	79	266	231	200	175	156	141	129	119	1.51	2.81	235	139	3.68	3.91
2	64	219	192	185	153	115	96	87	80	2.30	4.62	223	112	5.12	5.19
3	88	288	240	188	164	124	92	76	73	2.89	4.36	180	111	5.20	4.85
4	80	304	244	200	176	148	120	92	86	2.20	3.75	209	120	4.60	4.50
5	100–98	263	222	202	179	164	151	140	125	1.31	3.10	200	89	2.62	2.76
6	86–88	284	198	135	110	87	64	60	59	3.47	7.96	132	66	4.58	5.25
7	75–76	198	191	164	144	120	106	96	89	1.78	3.22	158	80	2.81	2.58
8	68–66	250	216	175	148	125	109	98	77	2.30	4.08	183	103	4.21	4.20
9	88–85	188	176	152	131	122	108	104	99	1.56	3.96	206	81	3.21	3.21
10	92–90	275	243	198	176	161	147	134	122	1.51	3.15	209	105	3.16	3.31
11	83–83	175	156	138	128	117	110	95	83	1.33	3.22	160	62	2.12	2.00
12	89–94	264	208	176	145	121	110	92	86	2.16	5.10	185	76	5.00	3.88
13	82–87	188	156	122	104	92	92	90	87	2.01	6.42	143	43	3.73	3.58
14	73–72	180	172	155	141	128	117	110	102	1.31	2.78	151	74	2.36	2.49
15	83–81	231	192	147	123	108	97	78	70	2.08	6.30	208	67	4.83	4.52
16	81–70	240	230	193	174	158	137	134	110	1.59	2.90	199	113	3.16	3.28
17	92–88	258	212	188	175	160	140	132	111	1.61	2.78	187	104	2.98	2.86
18	78–82	210	186	160	145	131	114	94	80	1.58	3.15	165	80	2.74	2.62
19	84–85	250	168	142	115	104	90	87	82	3.15	6.03	142	73	4.88	4.03
Average	83	238	202	164	148	129	113	101	92	1.97	4.20	180	89	3.74	3.68
S.D.	8	38	28	26	23	23	22	22	18	.62	1.52	29	24	1.04	0.93

* Diagnoses: Cases 1–5, 9, 11–13, 16–19: Volunteers. Cases 6, 7, 10, 14, 15: Fibroadenoma of breast. Case 8: Cervical erosion.

† Where single value for fasting glucose is given, only one fasting blood sample was obtained.

‡ Refer to text for definition of these terms and methods of calculating their values.

Procedures

All subjects were placed on a diet containing at least 225 grams of carbohydrate and 2400 calories daily for two weeks prior to study. Each individual, having fasted overnight for 14 hours, was kept completely at rest in an air conditioned room (temperature 78 to 81° F) for one-half hour prior to and during the test. An indwelling Cournand-type needle was placed in an antecubital vein. After two fasting blood samples had been obtained over a period of approximately 20 minutes, a 30 per cent glucose solution in distilled water was injected into another vein. In control subjects, 1 through 11, and patients 20 through 27, and 34 through 42, twenty-five grams of glucose was administered from a syringe over a four-minute period. In all other individuals, the glucose was administered using a Bowman constant infusion pump over a three to five-minute period. This permitted a more accurate determination of the amount of glucose delivered (Table VI).

Venous blood specimens were withdrawn without stasis into heparinized syringes at eight minutes following the start of the glucose injection and subsequently every eight minutes for 64 minutes. Glucose concentration and, when performed, serum inorganic phosphate and serum potassium concentrations were determined in duplicate on each blood sample. The blood specimens were immediately iced and protein-free filtrates for the glucose analyses were promptly prepared at the bedside. Urine was collected during the 75 minutes following the injection of glucose for the determination of urinary glucose excretion during the test.

Ten patients had 2 or 3 repeat glucose tolerance tests performed (Table III). Six of these subjects were maintained on a constant carbohydrate and caloric intake on the metabolic ward during the interval between the studies. The remaining four individuals followed the prescribed diet on an outpatient basis during the period of observation.

Glucose was determined in duplicate by the Nelson modification of the Somogyi method (19, 20). Serum inorganic phosphorus determinations were made according to the method of Taussky and Shorr (21). Serum potassiums were determined using a flame photometer with an internal standard.

Analysis of glucose tolerance curves

Methods of analysis of the rapid intravenous glucose tolerance test have varied. In the present study, each glucose tolerance curve was graphically evaluated both by the method suggested by Conard, Franckson, Bastenie, Kestens, and Kovacs (22) and by the technique employed by Amatuzio, Stutzman, Vanderbilt, and Nesbitt (23). These two methods were chosen because (a) they provide methods which facilitate analyzing the glucose tolerance curve in terms of a single constant, (b) a large amount of normal data is available in the literature for these techniques, and (c) in the present study, by means of statistical analyses which will be presented below,

TABLE II

Intravenous glucose tolerance tests in patients with neoplastic disease

Subject No.*	Fasting glucose†	Glucose concentration (*mgm. %*) (*Time in minutes*) 8	16	24	32	40	48	56	64	Disappearance rate constant (*%/min.*) Total	Increment	Mean glucose concentration‡ (*mgm. %*) Total	Increment	Net glucose disappearance‡ (*mgm. %/min.*) Total	Increment
20	84	237	215	183	169	163	152	145	129	0.75	1.84	222	99	1.67	1.82
21	104	305	255	227	212	196	181	170	—	1.08	2.12	231	120	2.49	2.54
22	90	324	260	220	196	172	152	141	112	1.51	2.89	231	127	3.49	3.67
23	93	218	194	168	152	144	134	127	120	0.88	2.78	187	74	1.65	2.06
24	90–94	256	228	217	194	190	174	166	157	0.78	1.61	228	121	1.78	1.95
25	82	322	268	228	180	166	140	108	84	2.02	3.07	221	138	4.47	4.23
26	101–98	255	230	218	200	192	171	163	132	0.91	1.60	207	116	1.88	1.89
27	96	309	260	233	212	182	170	157	142	1.22	2.43	244	133	2.98	3.23
28	56–59	219	192	180	168	158	147	137	120	0.79	1.26	184	118	1.48	1.52
29	84–86	342	296	272	240	218	200	183	166	1.25	2.02	270	177	2.97	3.15
30	98–93	282	258	236	224	210	195	180	170	0.90	1.44	227	157	2.36	2.60
31	119–115	312	288	270	250	228	218	206	195	0.94	1.61	269	130	2.56	2.11
32	84–81	285	240	210	190	169	151	137	124	1.33	2.43	220	128	2.99	3.17
33	80–81	309	264	231	209	192	176	162	150	1.17	2.06	243	149	2.91	3.13
34	75	200	187	171	157	138	126	116	99	1.23	2.49	172	86	2.11	2.14
35	86–88	242	192	178	156	143	132	116	98	1.15	2.71	171	80	1.97	2.17
36	120	363	321	302	261	242	210	206	195	1.06	1.80	267	152	2.83	2.74
37	90–86	204	180	169	160	147	135	128	112	0.94	2.19	174	79	1.64	1.73
38	80	239	192	164	140	133	121	109	95	1.26	3.05	179	81	2.26	2.47
39	110–114	239	224	212	204	199	192	180	170	0.51	1.27	213	99	1.09	1.26
40	78–79	245	206	180	170	145	137	128	107	1.17	2.31	186	100	2.17	2.31
41	108–106	282	257	242	226	205	200	185	176	0.80	1.85	342	99	2.73	1.83
42	92	334	226	218	207	189	178	163	154	0.82	1.73	287	126	2.34	2.18
43	110–106	252	238	225	212	201	191	183	175	0.67	1.57	237	115	1.61	1.83
44	84–38	240	200	190	176	164	159	148	141	0.68	1.49	234	128	1.69	2.02
45	92	196	164	146	142	112	110	90	86	1.26	4.62	173	52	2.24	2.48
46	75–78	294	250	210	180	162	141	114	102	1.82	3.08	221	132	4.02	4.07
47	78–80	279	251	208	197	182	169	159	149	0.98	1.61	193	128	1.89	2.05
48	65–68	185	171	152	138	123	118	101	92	1.15	2.39	154	82	1.58	1.75
49	77–81	198	170	155	146	139	129	123	117	0.73	1.73	154	66	1.00	1.00
50	78–74	311	244	185	157	124	107	96	87	2.36	5.22	218	106	5.76	6.20
51	84–84	264	228	217	204	198	189	180	172	0.53	1.02	213	126	1.13	1.29
52	87–89	192	177	165	153	145	137	131	119	0.75	1.76	169	62	1.41	1.22
53	90–91	204	181	162	147	140	126	119	115	1.10	2.73	173	67	2.63	2.67
54	87–89	246	218	194	184	166	152	141	129	1.12	2.28	196	101	2.27	2.36
55	91–92	242	222	203	189	175	168	154	146	1.02	1.88	199	105	2.07	2.01
Average	89	262	226	204	186	171	155	146	132	1.06	2.22	209	102	2.34	2.41
S.D.	13	46	40	34	32	31	30	30	31	0.33	0.85	40	9	0.91	0.96

* Diagnoses: Cases 20, 21, 24, 26–28, 30, 32, 33: Carcinoma of cervix, Stage I or II; Cases 22, 23, 29, 31: Carcinoma of breast; Case 25: Carcinoma of lung; Cases 39, 43, 49, 54, 55: Chronic myelogenous leukemia; Cases 34, 37, 53: Chronic lymphatic leukemia; Cases 35, 40–42, 44, 47: Hodgkins; Cases 46, 48, 50–52: Lymphosarcoma; Cases 36, 38, 45, 50: Reticulum cell sarcoma.

† Where single value for fasting glucose is given, only one fasting blood sample was obtained.

‡ Refer to text for definition of these items and methods of calculating their values.

they were found to provide good reproducibility in a given individual, and good discrimination between the control and neoplastic groups.

The intravenous glucose tolerance curve may be analyzed by plotting the log of the total blood glucose concentration against time to obtain a straight line (22). This relationship suggests that the rate of fall of blood glucose at any time is proportional to the total blood glucose value at that time.

Amatuzio and his co-workers (23) found that the plot of the log of the blood glucose value in excess of the initial fasting level against time was a straight line. These authors suggested, therefore, that the rate of fall of blood glucose at any time is proportional to the blood glucose concentration in excess of the fasting level at that time.

An index of glucose tolerance may be obtained by plotting the data according to either of the above indicated methods and then applying the equation:

$$K = \frac{\log_e 2}{t_{\frac{1}{2}}},$$

where in determining the fractional rate constant of disappearance of "total glucose" (K_b), $t_{1/2}$ is the time in minutes required for the total blood sugar value at any point to decline 50 per cent, or, in determining the fractional rate constant of disappearance of "increment glucose" (K_a), $t_{1/2}$ is the time in minutes required for the blood sugar value in excess of the fasting level at any point to decline 50 per cent.

Analysis of the present data, in accord with previously published studies (24, 25), indicates that neither of the

methods (22, 23) mentioned above describes the entire glucose tolerance curve. Thus, the values of blood glucose at eight minutes and at times when the concentrations of blood glucose were below 25 mg. per cent in excess of the fasting value generally deviated from the simple exponential relationship. In this study, the blood sugar values between 16 minutes and 56 minutes following the beginning of the glucose injection were employed for graphic analysis. In addition, K_b and K_a were calculated, by the method of least squares, for each glucose tolerance test.[4] The K values as determined 1) by this calculation and 2) by free-hand graphic analysis did not differ significantly, as shown by comparison of the means of the two paired samples.

The reliability of K_a and K_b in analysis of the present glucose tolerance data was further evaluated by two types of statistical analysis. First, employing the data obtained in repeat determinations in ten patients (Table III), analysis of variance revealed that both K_b and K_a showed a very small variation between repeat determinations in the same individual relative to the variation between individuals (Table IV). Amatuzio, Stutzman, Vanderbilt, and Nesbitt (23) have shown by duplicate tests in normal individuals that K_a is reproducible. Recently, Duncan (24) has demonstrated the reproducibility of both K_a and K_b in normal individuals. Secondly, employing the initial glucose tolerance test in each of the entire group of control and patient subjects, analysis of variance revealed that both K_b and K_a distinguished significantly between these groups (Table V). Furthermore, the discrimination between the neoplastic and normal group was equally good whether K_b or K_a was employed in analysis of the data (Table V).

It should be noted that the two methods employed in the analysis of the intravenous glucose tolerance curves are empirical and suggest different and mutually exclusive assumptions about the mechanisms controlling the rate of glucose disappearance. The present data indicate that while neither method is a completely accurate description of blood glucose disappearance, both fit the data sufficiently closely to provide good means of assessing glucose tolerance.

Net rate of disappearance of glucose. K_b and K_a provide a value for the fractional rate constant of glucose

[4] In actual practice, the constants were calculated in terms of the log to the base 10 and in terms of 8-minute periods. The constants (C) calculated in this manner were related to K_b or K_a by the equation:

$$2.3\ C = 8\ K$$

TABLE III

Repeat intravenous glucose tolerance tests in patients with neoplastic disease

Subject No.	Date	Fasting glucose*	Glucose concentration (*mgm. %*) (*Time in minutes*)								Disappearance rate constant (*%/min.*)	
			8	16	24	32	40	48	56	64	Total	Increment
41	6/22	108–106	282	257	242	226	205	200	185	176	0.80	1.85
	7/8	114–120	283	258	241	224	210	198	188	180	0.84	1.76
42	4/15	92	334	226	218	207	189	178	163	154	0.82	1.73
	4/21	96–100	276	231	196	186	187	174	161	150	0.82	1.87
43	5/6	110–106	252	238	225	212	201	191	183	175	0.67	1.57
	5/12	115–109	320	271	257	222	217	200	190	176	0.78	1.80
	5/19	118	278	258	241	226	212	201	197	185	0.76	1.60
44	7/6	86–86	240	200	190	176	164	159	148	141	0.68	1.49
	9/8	78–80	197	185	168	160	149	144	136	128	0.63	1.54
47	9/1	78–80	279	251	208	197	182	169	159	149	0.98	1.61
	1/12	86–84	300	288	256	233	215	199	184	170	1.02	1.61
48	9/12	65–68	185	171	152	138	123	118	101	92	1.15	2.39
	11/18	79–78	232	200	179	158	142	130	120	111	1.25	2.51
49	9/23	77–81	198	170	155	146	139	129	123	117	0.73	1.73
	1/12	82–90	264	218	201	191	180	168	158	147	0.80	1.57
52	11/21	87–89	192	177	165	153	145	137	131	121	0.75	1.76
	1/3	86–85	196	189	164	154	142	138	125	118	0.79	1.86
54	2/23	81–89	246	218	194	184	166	152	141	129	1.12	2.28
	3/1	79–82	228	192	177	162	151	136	121	112	1.12	2.15
	3/8	80–81	240	204	192	168	156	143	132	118	1.19	2.28
55	5/14	91–92	242	222	203	189	175	168	154	146	1.02	1.88
	5/24	90–92	264	240	222	206	184	175	164	150	1.02	2.01

* Where a single value for fasting glucose is given, only one fasting blood sample was obtained.

TABLE IV

Analysis of variance of indices of repeat glucose tolerance tests in ten patients with neoplastic disease

	Degrees of freedom	Total index: Sum of squares	Increment index: Sum of squares
Between individuals	9	.09951	.57087
Within individuals	10	.00263	.02477
Total	19	.10215	.59564
F*		41.92	25.61

$$*F = \frac{\text{Sum of Squares Between Individuals}}{\text{Sum of Squares Within Individuals}} \times \frac{10}{9}.$$

For the number of degrees of freedom present in this table, an F value greater than 4.95 would give a probability less than 0.01.

disappearance, *i.e.*, the per cent of glucose disappearing from the blood per minute. In an effort to evaluate the net amount of glucose disappearing from the blood, an estimate was made of the mean net rate of disappearance of glucose, *i.e.*, the milligrams of glucose disappearing per unit volume of blood per minute. The term net is used to indicate that this value represents the resultant of those reactions removing glucose from the blood and those reactions delivering glucose to the blood. In order to obtain the mean net rate of disappearance of "total glucose" from the blood per minute ($G.D._b$), the average "total glucose" concentration was multiplied by the rate constant K_b. The mean net rate of disappearance of "increment glucose" from the blood ($G.D._a$) was derived by multiplying K_a by the mean glucose concentration in excess of the fasting level.[5] All values for net rate of disappearance of glucose, in subjects receiving other than 25 gm. of glucose, were corrected to this dose. The value obtained for mean net rate of disappearance of glucose is defined by the method employed in its calculation. It is used in this study only for the purpose of comparing the control and neoplastic groups with respect to this parameter. The value for $G.D._b$ would be expected to be equal to the value for $G.D._a$ if both a plot of the log of the "total glucose" concentration against time and a plot of the log of the "increment glucose" concentration against time represent good approximations of a straight line.

Estimation of volume of distribution of glucose. An estimate of the volume of distribution of glucose (EVG) was obtained in those subjects in whom the glucose was given using a constant infusion pump to permit an accurate determination of the volume of glucose solution injected. The EVG was calculated by dividing the amount of glucose injected (mgm.) by the glucose concentration in excess of the fasting value (mgm. per 100 cc.) at the end of injection. This glucose concentration was determined by extrapolating to the end of the injection period the linear plot of the log of the glucose concentration in excess of the fasting level between 16 and 56 minutes following the injections. The values for blood sugar concentration prior to 16 minutes generally fell above this line and were not employed in this graphic analysis to reduce the error due to mixing. It is recognized that this calculation is not free from objection, particularly when applied to metabolizable substances. Nevertheless, it is assumed that these errors will be similar in both control and neoplastic subjects.

RESULTS

The data obtained from intravenous glucose tolerance tests in 19 control subjects and 36 patients with neoplastic diseases are presented in Tables I and II. The diagnosis of each subject is indicated in these tables.

Fractional rate constant of glucose disappearance

The fractional rate of "total glucose" disappearance (K_b) for the control group was 1.97 ±

TABLE V

Analysis of variance comparing the glucose tolerance tests in control subjects and patients with neoplastic disease

	Degrees of freedom	Total blood sugar index: Sum of squares	Increment blood sugar index: Sum of squares
Between groups	1	.64261	10.38503
Within groups	54	1.66644	27.77842
Total	55	2.30905	38.16345
F*		20.82	20.18

$$*F = \frac{\text{Sum of Squares Between Groups}}{\text{Sum of Squares Within Groups}} \times \frac{54}{1}.$$

For the number of degrees of freedom present in this table, an F value greater than 7.12 would give a probability less than 0.01.

[5] The value for the average "total glucose" concentration was obtained by the equation:

$$\bar{G}_b = \frac{G_o(1 - e^{-K_b t})}{t\,K_b},$$

where $\bar{G}_b$ is the average "total glucose" concentration in mgm. per 100 cc.; G_o is the total blood glucose concentration in mgm. per 100 cc. at zero time; t is the time interval in minutes during which the fractional rate of "total glucose" disappearance is constant.

The value for the average "increment glucose" concentration was obtained by the equation:

$$\bar{G}_a = \frac{(G_o - G_f)(1 - e^{-K_a t})}{t\,K_a},$$

where $\bar{G}_a$ is the average "increment glucose" concentration in mgm. per 100 cc.; G_f is the fasting blood glucose concentration in mgm. per 100 cc.; t is the time interval in minutes during which the fractional rate of "increment glucose" disappearance is constant.

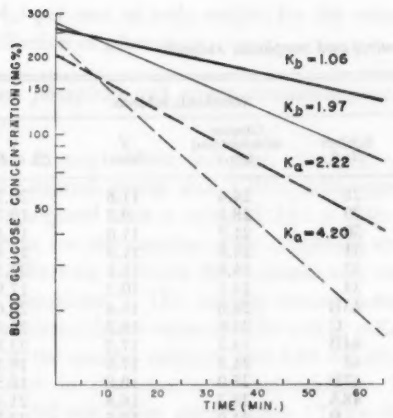

FIG. 1. THE AVERAGE CURVES OF TOTAL BLOOD GLUCOSE CONCENTRATION AGAINST TIME ARE PLOTTED FOR THE CONTROL (LIGHT SOLID LINE) AND PATIENT (HEAVY SOLID LINE) GROUPS AND THE AVERAGE CURVES OF THE BLOOD SUGAR CONCENTRATION IN EXCESS OF THE FASTING LEVEL AGAINST TIME ARE PLOTTED FOR THE CONTROL (LIGHT BROKEN LINE) AND PATIENT (HEAVY BROKEN LINE) GROUPS

The fractional rate of disappearance of "total glucose" (K_b) and the fractional rate of disappearance of "increment glucose" (K_a) are indicated in per cent per minute.

0.62 per cent per minute.[6] The K_b for the neoplastic group was 1.06 ± 0.33 per cent per minute. The fractional rate of "increment glucose" disappearance (K_a) in the control group averaged 4.20 ± 1.52 per cent per minute. The neoplastic group had a K_a of 2.22 ± 0.85 per cent per minute. The difference between the means for both K_a and K_b were statistically significant [7] (Figure 1). Examination of Tables I and II will reveal that K_b in five subjects with neoplastic disease and K_a in six of these patients fell within the range of values for the control series.

The data for the control subjects are in general agreement with previous results obtained using the type of intravenous glucose tolerance test employed in this study. In 60 normal subjects reported by Lozner, Winkler, Taylor, and Peters (26), the K_b was 1.96 ± 0.30 per cent per minute and the K_a was 3.57 ± 0.37 per cent per minute. Amatuzio, Stutzman, Vanderbilt, and Nesbitt (23) reported data on 70 control individuals with a K_b of 1.34 ± 0.27 per cent per minute and a K_a of 3.71 ± 0.40 per cent per minute. An average K_b of 1.52 ± 0.23 per cent per minute has been observed in 20 normal subjects (22). In 20 normal persons, Duncan (24) found a K_b of 1.37 ± 0.22 per cent per minute and a K_a of 3.68 ± 0.40 per cent per minute.

[6] Average values are given with one standard deviation.

[7] A significant difference between the means was taken to be a difference of at least 3.2 times the standard deviation of the difference between means ($p < .01$).

Blood sugar values

The fasting blood sugar for the control series was 83 ± 8 mgm. per cent and for the neoplastic group 89 ± 13 mgm. per cent (Tables I and II). The difference between these means was not statistically significant. Four patients with neoplastic disease did have fasting blood sugars in excess of 100 mgm. per cent, which was the upper value for the range of fasting blood sugar levels in the control group.

The average blood sugar values for the control and neoplastic groups at 8 and 16 minutes following glucose administration were not significantly different. The mean blood sugar concentrations at subsequent sampling times, *i.e.*, 24, 32, 40, 56 and 64 minutes, following glucose administration were significantly higher in the patients with neoplastic disease than in the control individuals (Tables I and II).

Urinary glucose excretion during the 75 minutes following the intravenous administration of glucose was determined in 14 control individuals and 22 patients. Urinary glucose excretion amounted to 0.8 ± 0.3 gm. during the 75-minute period in control individuals and 0.6 ± 0.3 gm. during the 75-minute period in the neoplastic group. There was no significant difference between these means.

Net rate of disappearance of glucose

The mean "total glucose" concentration ($\bar{G}_b$) was 180 ± 29 mgm. per cent in the control individuals and 209 ± 40 mgm. per cent in the neoplastic group. In the control group, the net rate of disappearance of "total glucose" from the blood ($G.D._b$) was 3.74 ± 1.04 mgm. per 100 cc. per minute compared with a value of 2.34 ± 0.91 mgm. per 100 cc. per minute for the individuals with neoplastic disease (Tables I and II). The difference between the mean $G.D._b$ and the difference

TABLE VI

Estimated glucose volume of distribution in control and neoplastic subjects

Control subjects				Neoplastic subjects			
Subject No.	Glucose administered (gm.)	V (liters)	V (% of BW)	Subject No.*	Glucose administered (gm.)	V (liters)	V (% of BW)
10	25.0	11.9	18.3	28	24.4	11.8	20.7
12	19.9	10.5	16.1	29	28.5	9.2	19.7
13	19.3	13.3	15.8	30	21.7	11.0	17.8
14	21.0	15.6	25.5	31	24.8	11.3	17.4
15	23.4	10.6	17.8	32	24.4	11.1	23.5
16	25.0	12.1	17.5	33	24.4	10.2	17.9
17	25.3	15.8	26.4	43B	24.6	16.4	26.6
18	23.7	14.8	21.2	C	24.6	16.2	26.3
19	22.9	11.8	16.6	44B	23.7	17.2	22.8
				45	24.2	17.0	19.1
Average	22.8	12.9	19.5	47B	25.0	10.8	18.5
S.D.		1.8	3.8	48A	28.2	16.8	21.4
				B	24.0	17.5	22.7
				49A	28.3	14.5	25.3
				B	25.1	15.5	26.6
				50	22.4	7.6	12.0
				51	25.0	15.6	26.4
				52A	22.4	15.7	23.8
				B	23.4	15.5	25.8
				53	17.1	12.8	17.3
				54A	24.2	16.1	25.5
				B	24.2	15.4	24.4
				C	25.3	15.2	24.1
				55A	24.6	13.3	21.8
				B	24.0	14.5	21.7
				Average	24.3	13.2	21.0
				S.D.		3.0	3.8

* Letters refer to repeat determinations at time of repeat glucose tolerance tests (Table III).

between the average G_b for the control and patient groups were statistically significant.

The mean "increment glucose" concentration ($\bar{G}_a$) for the control subjects had a value of 89 ± 24 mgm. per cent compared with 102 ± 9 mgm. per cent for the neoplastic patients. Net rate of disappearance of "increment glucose" ($G.D._a$) was 3.68 ± 0.93 mgm. per 100 cc. per minute for the control subjects and 2.41 ± 0.96 mgm. per 100 cc. per minute for the patients with neoplastic disease. The difference between the mean $G.D._a$ was significant, though there was no significant difference between the average values for $\bar{G}_a$ for the control and patient groups. There is close agreement between the values for $G.D._b$ and for $G.D._a$ in both the control and neoplastic groups as predicted from the mathematical considerations indicated in the method section.

Examination of Tables I and II will reveal that $G.D._b$ in 19 patients with malignant disease and $G.D._a$ in 24 of these subjects fell within the range of values for the control group.

Estimated volume of distribution of glucose

Estimation of the volume of distribution of glucose (EVG) (Table VI) in control subjects yielded a value of 12.9 ± 1.8 liters or 19.5 ± 3.8 per cent of body weight, and in the neoplastic group a value of 13.2 ± 3.0 liters or 21.0 ± 3.8 per cent of body weight. These averages revealed that the EVG did not differ significantly between the two groups.

The values for EVG in the present study are in accord with previous findings. Jokipii and Turpeinen (27), using a 60-minute constant glucose infusion technique, reported a volume of distribution of glucose that averaged 17.0 per cent of body weight. One method (28), involving a single injection of carbon 14 labelled glucose, yielded an average volume of distribution of glucose of 24 per cent of body weight. Employing the same method as used in the present study, Dominguez (29) reported an average value of 19.0 per cent of body weight and Conard (30) found a mean

of 24.3 per cent of body weight for the volume of distribution of glucose.

Serum phosphate and serum potassium concentration

The fasting serum inorganic phosphate level in the control group was 3.19 ± 0.36 mgm. per cent compared with a value of 3.61 ± 0.48 mgm. per cent for the patients with neoplastic disease. The difference between these means was statistically significant. The fasting serum potassium concentration had a value of 4.34 ± 0.32 mEq. per liter in the control subjects and 4.45 ±0.34 mEq. per liter in the neoplastic individuals. These means did not differ significantly (Table VIII).

Most investigators consider the maximum fall in serum inorganic phosphate level and the maximum fall in serum potassium concentration as the important determinations in terms of reflecting glucose utilization, and the present data were analyzed from this standpoint. The maximum fall in serum inorganic phosphate in the control group was 0.67 ± 0.19 mgm. per cent or 22.0 ± 6.2 per cent of the fasting level. In the neoplastic subjects, the maximum decrease in serum inorganic phosphate was 0.61 ± 0.24 mgm. per cent or 16.7 ± 5.7 per cent of the fasting concentration. The difference between the mean values for the maximum fall of serum phosphate expressed in absolute terms was not significant. However, when the fall in serum phosphate concentration was expressed as a per cent of the fasting level, the difference between the means was of borderline significance. After a rapid injection of 25 gm. of glucose, Wertheim, Eurman, and Kalinsky (31) found a maximum fall in normals of 0.58 ±

TABLE VII

Serum inorganic phosphorus (mgm. %) and potassium (mEq./L.) concentrations during intravenous glucose tolerance tests

Control subjects							Neoplastic subjects						
	Serum phosphate			Serum potassium				Serum phosphate			Serum potassium		
Subject No.	Fasting	Max. decrease	% Decrease	Fasting	Max. decrease	% Decrease	Subject No.	Fasting	Max. decrease	% Decrease	Fasting	Max. decrease	% Decrease
1	3.53	0.93	26.4	5.00	0.88	17.6	20	3.30	0.60	18.2	3.63	0.44	12.1
2	3.30	0.31	9.4	3.78	0.08	21.2	21	3.71	0.52	14.0			
3	3.38	0.90	26.8	4.23	0.15	3.5	22	3.66	1.06	28.9	4.25	0.63	14.8
4	2.75	0.65	23.6	4.72	0.30	6.4	23	3.98	0.48	12.1	4.56	0.21	4.6
5	3.25	0.61	18.8	4.60	0.87	18.9	24	3.82	0.55	14.4	4.27	1.07	25.1
6	2.67	0.88	33.0	4.19	0.33	7.9	25	4.05	0.85	21.0			
7	2.83	0.83	29.4	4.06	0.50	12.3	26	3.15	0.65	20.6			
8	3.47	0.93	26.8	3.98	0.49	12.3	27	2.87	0.54	18.8	4.30	0.40	9.3
9	3.60	0.80	22.2	4.34	0.12	2.8	28	4.42	0.77	17.4	4.68	0.62	13.2
10	3.62	0.73	20.2	4.49	0.59	13.1	29	3.45	0.53	15.4	4.33	0.23	5.3
11	2.73	0.61	22.4	4.46	0.24	5.4	30	3.54	0.76	21.5			
12	2.69	0.49	18.2	4.20	0.33	7.9	31	3.28	0.37	11.3	4.60	0.55	12.0
13	3.27	0.54	16.5				32	4.05	0.90	22.2			
14	3.62	0.52	14.4				34	3.35	0.60	17.9	4.40	0.59	13.4
							35	3.10	0.29	9.3			
Aver.	3.19	0.67	22.0	4.34	0.41	10.8	36	2.26	0.38	16.8	4.18	0.89	21.2
S.D.	0.36	0.19	6.2	0.32	0.28	5.9	37	3.14	0.30	9.6			
							38	3.77	0.88	23.3	4.14	0.51	12.3
							39	4.21	1.14	27.1	5.15	1.33	25.8
							40	3.85	0.95	24.6			
							41	3.60	0.50	13.9	4.79	0.21	4.4
							42	3.95	0.55	13.9	4.66	0.68	14.6
							43	4.10	0.52	12.7	3.92	0.44	11.2
							44	3.87	0.58	15.0	4.96	0.48	8.3
							45	4.43	0.33	7.4			
							47	4.20	1.06	25.2	4.16	0.37	8.9
							48	3.78	0.18	4.8	5.10	0.79	15.5
							49	4.03	0.52	12.9			
							50	3.19	0.54	16.9			
							52	3.09	0.42	13.6			
							53	3.40	0.60	17.6			
							Aver.	3.61	0.61	16.7	4.45	0.59	13.5
							S.D.	0.48	0.24	5.7	0.34	0.32	7.5

0.24 mgm. per cent or 16.8 ± 5.9 per cent of the fasting value, and Lazarus, Volk, Jacobi, and Gilady (32) reported a mean maximum fall in normals of 14.2 per cent (10 to 22.5 per cent). These changes are more comparable to the findings in the patient group than in the normal individuals of the present study. It should be noted that in previous reports as well as in the present data, the range of values for maximum fall in serum inorganic phosphate is large.

The serum potassium concentration had a maximum fall of 0.41 ± 0.28 mEq. per L. or 10.8 ± 5.9 per cent of the fasting level in the control individuals. In the patient group, the maximum decrease in serum potassium was 0.59 ± 0.32 mEq. per L. or 13.5 ± 7.5 per cent of the fasting concentration. There was no statistically significant difference between the mean values for maximum fall, expressed in absolute or relative terms, in serum potassium concentration.

DISCUSSION

A decrease in glucose tolerance, as indicated by a slower fractional rate of disappearance of blood glucose, was found to be associated with the presence of malignant disease. The fasting blood sugar was within the normal range in these subjects. The group of patients studied included individuals with lymphoma, chronic leukemia, and clinically localized carcinoma of the cervix, and carcinoma of the breast. These individuals were carefully selected to exclude various other factors known to impair carbohydrate metabolism.

The slower fractional rate of disappearance of glucose in the patients with malignant disease was associated with a higher mean blood sugar concentration following the intravenous administration of glucose. The question arises, in view of these findings, as to whether the net amount of glucose disappearing from the blood in the neoplastic group differed from the control subjects. The mean value for the net rate of disappearance of glucose was significantly lower in the patients compared with the control group. However, in the patient group, a normal net rate of disappearance of glucose was more frequently observed than a normal fractional rate of disappearance of glucose. There was no difference in the estimated volume of distribution of glucose between the patients and the control individuals. These calculated values derived from the disappearance curve of glucose indicate that in certain neoplastic subjects, despite a decreased fractional rate of disappearance of glucose, the persistence of blood glucose concentrations at significantly higher levels than in normal subjects may be associated with an approximately normal net rate of disappearance of glucose. A possible explanation of these findings is that net hepatic glucose output during the hyperglycemic phase is greater in the patients than in the control individuals and contributes to maintaining the higher blood glucose concentration. This explanation does not exclude a concomitant decrease in tissue glucose utilization in these patients.

The present data do not permit conclusions as to the mechanism of the decrease in glucose tolerance. A slower fractional rate of disappearance of glucose, whether or not associated with a decreased net rate of disappearance of glucose, might result from an alteration in hepatic glucose metabolism, a decrease in peripheral utilization, or a disturbance in both of these metabolic processes.

Peripheral glucose utilization has been found to be associated with a fall in serum inorganic phosphate following glucose administration (33, 34). A decrease in serum potassium concentration has also been reported to parallel glucose utilization (35). No statistically significant difference was observed between the control and patient groups with reference to the maximum fall in the absolute concentration of serum inorganic phosphate or serum potassium. The mean fasting serum inorganic phosphate concentration in the patients with malignant disease was higher than in the control subjects. The maximum fall in serum inorganic phosphate expressed as per cent of the fasting level was smaller in the patient group than in the control individuals. It should be noted that in the patients with malignant disease, the maximum decrease in serum inorganic phosphate concentration and in serum potassium concentration was found to be variable, and in any given individual the magnitude of fall of these two ions did not parallel each other. The meaning of these findings in terms of glucose metabolism is not apparent.

The fact that the decrease in glucose tolerance

was associated with localized carcinoma, small in size relative to total body mass, would make it unlikely that, in these patients at least, the decreased rate of disappearance of glucose was attributable to the carbohydrate metabolism of the tumor. Indeed, Cori and Cori (36) demonstrated that tumor *in vivo* had an increased rate of glycolysis. It would appear that the defect in glucose metabolism reflects alterations in host tissue metabolism associated with the presence of the neoplastic process. In experimental animals, there are several reports of defects in enzyme activity (37), including enzymes involved in carbohydrate metabolism (38), of tissues of the tumor-bearing host.

There are many studies on the incidence of cancer in diabetes mellitus (39). In general, these reports found that cancer occurs more frequently than expected in diabetics. The pertinence of this finding, if real, to the present data cannot be evaluated on the basis of available knowledge. The possibility does exist that the decrease in glucose tolerance in patients with malignant disease may reflect latent diabetes mellitus.

It should be emphasized that the defect in carbohydrate metabolism, as measured by the intravenous glucose tolerance test, is not at all specific. A decrease in glucose tolerance has been observed in a wide variety of conditions in addition to diabetes mellitus, such as endocrine disorders, hepatic disease, malnutrition, infectious diseases, neuropsychiatric disorders, renal disease and obesity. None of these conditions existed in the present group of patients with neoplastic disease. Thus, this defect in glucose metabolism can be considered of no diagnostic value; however, its definition may shed some light on the metabolic alterations associated with neoplasia, as well as other conditions in which a decreased glucose tolerance may be present.

SUMMARY

1. Intravenous glucose tolerance tests were performed in 36 carefully selected patients with chronic leukemia, lymphoma, and clinically early epithelial neoplasms, and in 19 subjects without cancer.

2. In analyzing the glucose tolerance curves, plotting either the log of the total blood sugar concentration against time, or the log of the blood sugar concentration in excess of the fasting value against time, provided an equally good index of glucose tolerance.

3. The patients with malignant disease compared with the control subjects had a significantly decreased fractional rate of disappearance of glucose and a significantly lower net rate of disappearance of glucose.

4. The fasting blood sugar concentration of the control and neoplastic groups did not differ significantly.

5. No significant difference in the estimated volume distribution of glucose was found between the two groups.

6. In the patients with malignant disease, the fasting serum inorganic phosphate concentration was significantly greater and the maximum per cent fall in inorganic phosphate was significantly less than in the control individuals. No significant difference was observed in the fall in serum potassium concentration between the two groups.

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ABSORPTION OF WATER AND SODIUM FROM THE SMALL INTESTINE OF PATIENTS WITH NONTROPICAL SPRUE

By JOHN A. HIGGINS, PHILIP R. LEE, JOHN F. SCHOLER, RICHARD J. REITEMEIER, CHARLES F. CODE, AND ERIC E. WOLLAEGER

(From the Sections of Medicine and Physiology, Mayo Clinic and Mayo Foundation, Rochester, Minn.)

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There is evidence that the absorption of water from the gastrointestinal tract of patients with nontropical sprue is delayed when they have recently eaten food (1) and also when they have fasted (2, 3). Excessive fecal loss of sodium has been demonstrated in patients with nontropical sprue (4), and recently evidence obtained by the use of isotopic sodium has indicated that the absorption of sodium chloride from the small bowel of patients with this disease is delayed (5).

The present study was undertaken to confirm, if possible, the presence of a defect in the absorption of water or sodium in fasting patients with nontropical sprue and to obtain a more exact measurement of its degree. The study was made possible by the recent development of a method that allows the precise quantitative determination of the rate of absorption of isotopically labeled substances from the gastrointestinal tract of human beings under completely physiologic conditions (6).

METHODS

Fourteen patients who presented typical clinical, laboratory and roentgenographic features of nontropical sprue were studied. Three were men and 11 were women. Their ages ranged from 23 to 63 years with the majority being in the mid-thirties. Rates of absorption were determined in most of the patients on only one occasion, the majority of these being obtained during relapses of the disease. Six tests were made during remissions.

A designation of the clinical status of the disease in each patient at the time the tests were performed has been used. When loss of weight, abdominal discomfort and diarrhea or any combination of such features of the disease predominated, the patient's clinical status was termed a "relapse." When such features were absent, or nearly so, the term "remission" has been used. These terms are intended to provide only a very rough estimate of the severity of the disease in each of the patients at the time the test of absorption was made.

All observations were made in the morning. The patients had eaten nothing since the meal of the previous evening. In each test the isotopes were introduced into the upper part of the small bowel through a Sawyer tube after the position of the tube in the third portion of the duodenum had first been established by fluoroscopic examination. As a routine, about 10 gm. of barium sulfate was mixed with the test material and its distribution throughout the abdomen followed by frequent fluoroscopic and roentgenographic observation.

The tests made in the course of this study fall into two groups. In the first, the rate of absorption of water alone was measured; eight such observations were made on five patients. In the second group, the rates of absorption of both water and sodium were determined. In addition to the dual determinations, refinements in methodology developed during the progress of the second part of the study allowed more precise determination of the rates of absorption of both water and sodium.

The method (6) employed for estimation of the rate of absorption of a labeled substance requires determination of its rate of appearance in the arterial blood while it is being absorbed, as well as its rate of disappearance from the arterial blood stream after its rapid intravenous injection. The precise rate of absorption of the isotope is then calculated by integration of these two rates. In the past it has been necessary to employ mean rates of arterial disappearance of the isotopes as determined in a group of healthy persons (6-8), and this was the procedure adopted in the first group of observations presented in this report. While use of a mean rate of arterial disappearance has been appropriate for the study of normal human beings, since rates of arterial disappearance vary so little among healthy persons, it was recognized at the outset that it might not be as applicable to the study of patients who are sick. The first step toward correction of this weakness was to determine the rate of disappearance of each isotope following its intravenous injection a few days before, or after the determination of its rate of appearance in the arterial blood during absorption from the bowel. This procedure was adopted in four of the nine patients of the second series; a further improvement was made in the tests on the five remaining patients. In these, dual isotopes of water (deuterium and tritium oxide) and dual isotopes of radiosodium (sodium^{22} and sodium^{24}) were employed (9). One of the isotopes of each pair was given intravenously while

simultaneously the other of each pair was placed in the small bowel. Determination of the concentration of the isotopes in the same samples of arterial blood then yielded simultaneous appearance and disappearance rates for both water and sodium. These rates were then employed in the calculation of the rate of absorption of each of the isotopes. The procedures followed in carrying out these tests did not differ significantly from those followed when single isotopes were used (6). The methods followed in analyzing for the dual isotopes in the blood and the validity of the procedures were first established by experiments on dogs and then applied to tests on human beings (9).

The quantities of the isotopes used in the test ranged from 19 to 50 gm. of deuterium oxide (D_2O), 2.5 to 3.0 millicuries of tritium oxide, 10 to 25 microcuries of radiosodium[22] and 14 to 100 microcuries of radiosodium[24]. The radiosodium was routinely dissolved in the labeled water and sufficient sodium chloride was added to make the solution isotonic. Zero time in the test was taken as the midpoint of the injection of the isotopes, those into the bowel being synchronized with those into the vein. Samples of arterial blood were drawn at minute intervals for 12 minutes and then less frequently until the termination of the test at 1 to 2 hours. Samples of blood for determination of the 3-hour or 24-hour equilibrium values were drawn by separate venipuncture. The concentrations of the isotopes in the blood were determined according to methods that have been described previously [deuterium with a mass spectrometer (10, 11), tritium with a liquid scintillation counter (12) and radiosodium in a well-type sodium iodide (thallium) scintillation counter].

Calculation of the rates of absorption of the isotopes was carried out according to the procedure previously described (6) except that in the last group of tests the time intervals used in the calculations were changed. The concentrations of the isotopes at 1½, 3½, 4½, 5½ minutes and so on, up to 11½ minutes, and the concentrations at 14 minutes and every 4 minutes thereafter up to 50 and then every 10 minutes for the remainder of the first 1½ hours were employed instead of those previously reported. This represents a slightly different time scheduling from that employed previously in the calculation. The change has been found to improve the determination of the rate of absorption of sodium. Such rather minor factors have been brought into focus by elimination of the use of a mean disappearance curve and adoption of the more accurate procedure of using the disappearance curve belonging to each individual, particularly when this is determined at the same time as the test of absorption. The concentration of the labeled water in the venous blood 3 hours after its administration was used as the equilibrium value in the calculations. In the case of sodium, the concentration in the venous blood at 24 hours was used as the equilibrium value in the majority of the tests; use of a 3-hour to 9-hour equilibrium in a few instances did not significantly alter the results. In each determination the percentages of the isotopes absorbed as the test progressed were plotted, and the slope of the straight line that best fitted the points which included the absorption of at least the first 50 per cent of the isotopes was expressed as the initial rate of absorption; in addition, the time required for the absorption of the first 50 per cent and 67 per cent of the administered amount of each of the isotopes was recorded.

RESULTS

Patients in whom absorption of water alone was tested

In this group of tests a mean rate of disappearance of deuterium oxide from arterial blood as determined in a series of normal persons was used in the calculation of the rate of absorption. The results may be compared directly with those obtained previously in our laboratory with the same technic (6–8). We have calculated the mean values for the total of 29 healthy persons tested in

TABLE I

Group 1—Rate of absorption of water from small bowel of patients with sprue

Case	Test	Initial rate, % absorbed per minute	Minutes required for absorption of		Clinical status
			50%	67%	
1	126	21.7	2.8	4.7	In remission
	113	13.5	4.4	6.4	In remission; relapsed 5 days later
2	119	7.1	6.8	12.8	In relapse
3	125	7.8*	6.4	9.6	In relapse
	112	10.0	5.3	9.1	Recovering from relapse
4	74	10.9	4.9	6.9	Recovering from relapse
5	147	14.7	4.3	6.4	In remission
Controls†		23±6	2.8±0.7	4.6±1.3	

* Hypertonic solution placed in bowel.

† Series of 29 persons from studies by Lee, Code, and Scholer (7) and by Reitemeier, Code, and Orvis (8); values following the ± sign are the standard deviations of the means.

TABLE II

Group 2—Rate of absorption of water from small bowel of patients with sprue

Case	Test	Initial rate, % absorbed per minute	Minutes required for absorption of		Clinical status
			50%	67%	
6	234	6.4	7.5	13.1	Relapse
7	271	7.1	6.9	10.8	Relapse
9	274	9.4	5.5	9.2	Relapse
10	259	10.4	5.3	10.4	Remission
11	198	20.0	3.1	4.0	Recovering from relapse
12	236	21.7	2.3	3.1	Recovering from relapse
13	254	25.0	2.7	3.4	Remission
14	262	26.3	1.9	2.6	Remission

these earlier studies; the mean initial rate of water absorption, per minute, was 23 per cent of the administered water (standard error of mean 1.1); the slowest rate was 14.8 per cent per minute. All of the patients with sprue who were in relapse had rates that were slower than this (Table I). In general, the rate of water absorption paralleled the clinical condition of the patient, the slowest rates of absorption being encountered in patients in relapse and the most rapid rates in those recovering or in remission. This generalization was also reflected in the time required for absorption of 50 per cent and 67 per cent of the administered water.

Patients in whom rates of absorption of both water and sodium were tested simultaneously

In this group of tests the rates of disappearance of water and sodium from arterial blood were determined individually for each patient, thereby eliminating the use of a mean disappearance rate in the calculation of the rate of absorption. The initial rates of absorption of water in three healthy persons using this refinement in technic were 16, 21 and 24 per cent per minute, which values are within the ranges of those obtained in normal persons with the less accurate procedure. Once again, in this more accurate series of observations, all of the patients in relapse had rates of absorption of water that were slower than those of normal persons and there was, in general, a parallelism between the rate of absorption of water from the small bowel and the condition of the patient (Table II). The combined results of the two groups of observations allow the general conclusion that the rate of absorption of water from the small bowel of patients with sprue while in relapse and under fasting conditions is slower than that of healthy persons, but when the patients are in a remission the rate approaches or becomes equal to that of normal persons.

The rate of absorption of sodium from the small bowel of the patients with sprue was even more decisively retarded than was the absorption of water. Tests of the rate of absorption of sodium have been made on 12 healthy persons in the course of other studies (unpublished data). The mean initial rate of absorption from the small bowel of the group was 9.5 per cent per minute and the slowest rate encountered was 6 per cent per minute. Only one of the patients with sprue had a rate of absorption that fell within the normal range, and this patient had a very mild form of the disease and was in remission at the time of the test (case 14, Table III). All of the patients studied during a relapse had exceedingly slow rates of absorption, ranging from 0.6 to 3.8 per cent per minute, and once again there was a rough correlation between the degree of impairment of the absorption of sodium and the clinical condition of the patient.

Hypomotility

The 10 gm. of barium sulfate suspended in the labeled water and placed in the small bowel did not allow a very precise estimate of motility of the small bowel but it was sufficient to demonstrate the decisive roentgenographic difference shown previously by others between sprue patients in relapse and healthy persons. In the patients, the barium spread very slowly through the abdomen and often remained "puddled" in the upper part

TABLE III

Group 2—Rate of absorption of sodium from small bowel of patients with sprue

Case	Test	Initial rate, % absorbed per minute	Minutes required for absorption of		Clinical status
			50%	67%	
6	234	0.6	80.0	112.5	Relapse
7	271	0.7	>85.0		Relapse
8	188	3.8	21.8	49.7	Relapse
9	274	0.8	>85.0		Relapse
10	259	0.7	97.0	115.0	Remission
11	198	4.0	15.4	35.5	Recovering from relapse
12	236	2.8	18.7	29.2	Recovering from relapse
13	254	5.7	10.1	14.0	Remission
14	262	10.4	4.6	6.5	Remission
Mean of 12 healthy persons*		9.5	6.3	8.0	

* Series from study by Reitemeier, Code, and Orvis (8).

of the small bowel throughout most of the test, whereas in healthy persons the same amount of barium suspension was quickly distributed over a wide area.

DISCUSSION

It should be emphasized at the outset that intestinal absorption as determined by the technics employed in this study is the unidirectional passage of the labeled material from the lumen of the bowel to the arterial blood. It is not a measure of the net result of exchanges in both directions across the intestinal mucosa which has often been used by others as the index of absorption.

Wollaeger and Scribner (1) found that absorption of water was retarded in patients with sprue when the water was taken during or following a meal. The results obtained in the present study demonstrate that the absorption of water is retarded in patients with sprue even in the absence of food. These results confirm the earlier findings of Taylor (3). They indicate in addition that the degree of retardation parallels in a rough way the condition of the patient, being greatest during exacerbations of the disease. The present study also confirms the findings of Newsholme and French (5) that there is a delay in the absorption of sodium from the small intestine of patients with sprue. The degree of the defect of absorption is greater for sodium than for water. The question arises, "Is sodium the anchor that holds the water in the bowel?" It seems likely that other factors are also involved.

Although this investigation demonstrates slowed absorption of water and sodium from the small bowel of patients with nontropical sprue, it does not define the mechanism of the defect. Reduced motility was certainly present in the small bowel of all of our patients in relapse. It has been shown by others that the absorption of glucose, methionine (13) and vitamin A (14) from the small bowel of human beings is slowed or hastened by decreases or increases in the motor action of the small intestine. Higgins, Code, and Orvis (15) have recently found that the absorption of water and sodium from the upper part of the small bowel is retarded in healthy persons when propulsive motility is reduced or eliminated by the administration of methantheline bromide. It seems likely that the reduced motility of the small bowel of the patients we studied contributed to the slowed absorption of both water and sodium. Other factors, however, were most likely also involved, for the much greater retardation of the absorption of sodium than of water suggests additional and more specific defects such as might occur in the membrane or membranes separating blood from bowel contents.

SUMMARY

The rate of absorption of isotopically labeled water and sodium from the small bowel in 14 patients with nontropical sprue was abnormally slow when the patients were tested during relapse of their disease. When the patients were tested during remission, the rates approached or became equal to those of healthy persons.

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ADAPTIVE VALUE OF RESPIRATORY ADJUSTMENTS TO SHUNT HYPOXIA AND TO ALTITUDE HYPOXIA[1]

By GEORGE HUSSON[2] AND A. B. OTIS[3]

(From the Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, Md.)

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Only a few adaptive mechanisms by which the body responds to hypoxia are at present understood well enough to be quantitatively examined and evaluated. Among these are the alterations that occur in the pulmonary ventilation, the acid base balance of the blood and the oxygen carrying capacity of the blood. It is the purpose of this paper to compare the adaptive value of these adjustments, as judged by their effectiveness in raising the partial pressure of oxygen in blood and tissues, in two types of hypoxia 1) that arising from chronic exposures to a low P_{O_2} in the environment (altitude hypoxia) and 2) that arising from the presence of a right to left shunt in the circulation (shunt hypoxia). We shall attempt to show that although the changes which occur in these two types of hypoxia are qualitatively similar in some respects, there are important quantitative differences in their adaptive value. We shall also present evidence that in the case of shunt hypoxia there is no reduction in the basal oxygen requirement of the body. For previous work by other authors pertinent to our general subject see references 1 through 4. More detailed data and a description of methods used in this investigation may be found in references 5 through 8.

Pulmonary ventilation

Numerous investigators have shown that individuals who are chronically hypoxic from prolonged residence at high altitudes have an increased ventilation which is greater the higher the altitude. There is evidence in the literature that individuals who are hypoxic because of circulatory shunts also ventilate more than normal.

We have measured the total resting ventilation of individuals with shunt hypoxia and have confirmed the presence of hyperventilation (6). In a smaller number we have made measurements of the resting alveolar ventilation. The results are shown as points in Figure 1. For comparison a curve previously published by Rahn and Otis (9) and indicating the magnitude of alveolar ventilation as a function of alveolar P_{O_2} in healthy individuals who had lived at altitude for relatively extended periods is also shown. Although our data show a rather large variation among individuals

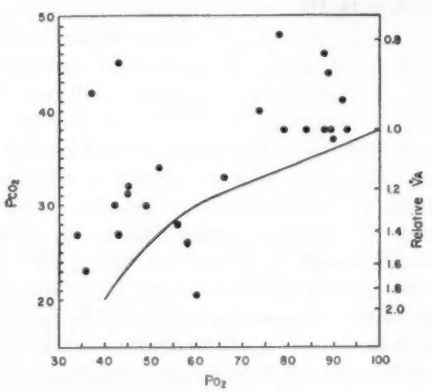

FIG. 1. ALVEOLAR P_{CO_2} AND RELATIVE ALVEOLAR VENTILATION AS A FUNCTION OF ARTERIAL P_{O_2}

The solid circles represent measurements made on patients with congenital heart disease. Those with a P_{O_2} less than 75 mm. had right to left circulatory shunts, those with a P_{O_2} greater than 75 mm. did not. The alveolar P_{CO_2} was either directly determined from analysis of end tidal samples or calculated from expired P_{CO_2} and estimated dead space. The curve is taken from Rahn and Otis (9) and applies to normal individuals acclimatized to various altitudes. Relative alveolar ventilation was calculated as follows:

$$\dot{V}_A = \frac{38 \text{ mm.}}{P_{A}^{\cdot}{}_{CO_2}}$$

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[2] Present address: Department of Pediatrics, Syracuse Memorial Hospital, Syracuse 10, New York.

[3] Present address: College of Medicine, University of Florida, Gainesville, Florida.

they demonstrate a definite tendency for the alveolar ventilation to increase with decreasing arterial P_{O_2}, the relationship being similar in form but less in extent to that found for the altitude dwellers.

The hyperpnea of the altitude dweller constitutes for him an important adaptation. If at 15,000 feet, for example, an individual did not increase his ventilation above the sea level value, his alveolar (and arterial) P_{O_2} would be about 38 mm. Hg. His arterial oxygen saturation would be about 72 per cent. The increase in ventilation that usually occurs in the individual acclimatized to this altitude is such as to increase his arterial P_{O_2} to about 50 mm. Hg and his arterial saturation to about 83 per cent, an increase which is really significant as an adaptive mechanism.

The victim of shunt hypoxia on the other hand, can increase the oxygenation of his arterial blood but little by hyperpnea. His pulmonary venous blood will be about 97 per cent saturated even with a normal ventilation; and although increasing the ventilation will raise the P_{O_2} of this blood, it will alter the per cent saturation only insignificantly be-

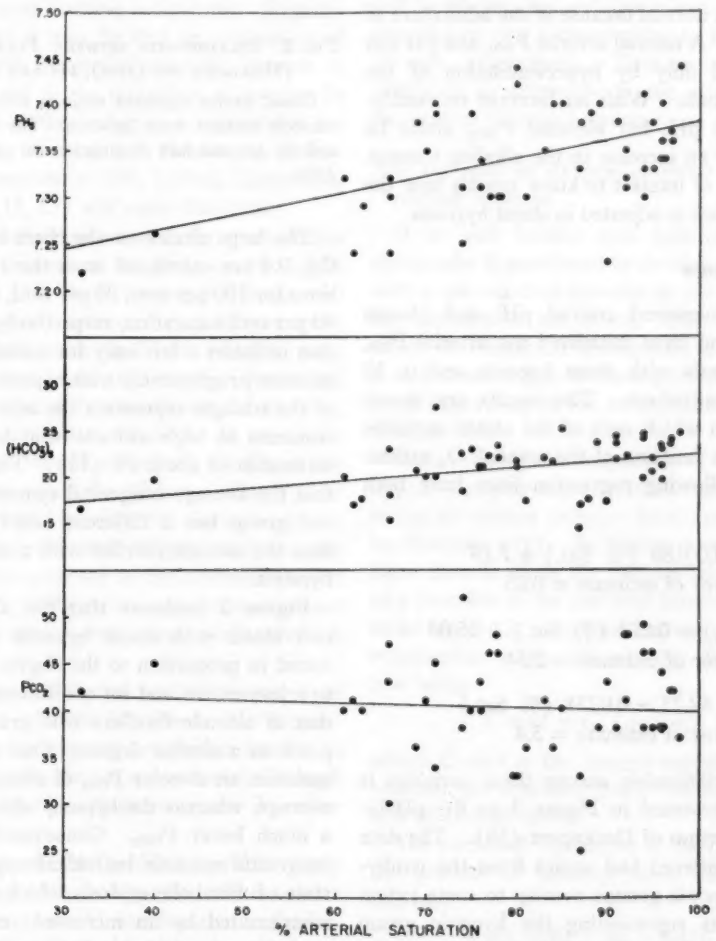

FIG. 2. PLASMA pH, PLASMA BICARBONATE CONCENTRATION IN MILLIMOLS PER LITER AND PARTIAL PRESSURE OF CARBON DIOXIDE IN ARTERIAL BLOOD AS A FUNCTION OF ARTERIAL OXYGEN SATURATION

cause of the flatness of the dissociation curve in this region. Furthermore that component of this mixed arterial blood which is shunted around his lungs will not be directly affected at all. Resting hyperpnea in such an individual would therefore seem to be of negligible adaptive value as far as improvement of oxygenation of his systemic arterial blood is concerned.

However, the presence of a right to left shunt introduces a problem in CO_2 elimination as well as of oxygen uptake. With a normal resting ventilation the pulmonary venous P_{CO_2} will be normal but the arterial P_{CO_2} will be higher and arterial pH lower than normal because of the admixture of venous blood. A normal arterial P_{CO_2} and pH can be maintained only by hyperventilation of the proper magnitude. With no increase in ventilation a normal pH, but elevated P_{CO_2}, could be maintained by an increase in the alkaline reserve. It is therefore of interest to know exactly how the acid base balance is adjusted in shunt hypoxia.

Acid base balance

We have measured arterial pH, and plasma bicarbonate and have calculated the arterial P_{CO_2} in 35 individuals with shunt hypoxia and in 12 nonhypoxic individuals. The results are shown in Figure 2 in which each of the above variables is plotted as a function of the arterial O_2 saturation. The following regression lines have been calculated.

$$pH = 0.00189\ (\%\ \text{Sat.}) + 7.19$$
Std. error of estimate = 0.05

$$(HCO_3) = 0.074\ (\%\ \text{Sat.}) + 15.09$$
Std. error of estimate = 2.54

$$P_{CO_2} = 42.23 - 0.0234\ (\%\ \text{Sat.})$$
Std. error of estimate = 5.4

The interrelationship among these variables is graphically presented in Figure 3 on the pH-bicarbonate diagram of Davenport (10). The data are widely scattered and values from the nonhypoxic and hypoxic groups overlap to some extent but the points representing the hypoxic group tend to fall in the region of the chart below the normal pH and below the normal buffer line, and so indicate metabolic acidosis.

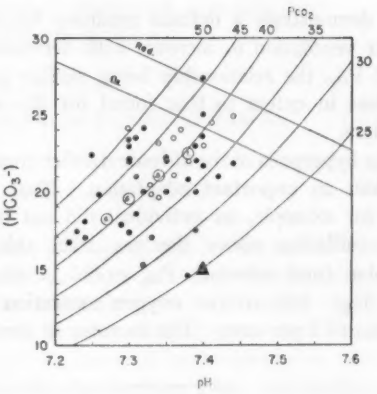

FIG. 3. RELATIONSHIP BETWEEN PLASMA BICARBONATE (MILLIMOLS PER LITER), pH AND P_{CO_2} MM. HG

Closed circles represent subjects with and open circles subjects without shunt hypoxia. The lines labelled red. and ox. are standard dissociation curves from Davenport (10).

The large circles on the chart labelled 1.0, 0.8, 0.6, 0.4 are calculated from the regression equations for 100 per cent, 80 per cent, 60 per cent, and 40 per cent saturation, respectively, and their position indicates a tendency for metabolic acidosis to increase progressively with hypoxia. The position of the triangle represents the acid-base picture of residents at high altitude who have a per cent saturation of about 75 (11). There is no doubt that the average congenital cyanotic individual in our group has a different acid-base adjustment than the altitude dweller with a similar degree of hypoxia.

Figure 2 indicates that the alkali reserve of individuals with shunt hypoxia tends to be reduced in proportion to the degree of hypoxia but to a less extent and for a different reason than is that of altitude dwellers who are chronically hypoxic to a similar degree. Our subjects tend to maintain an alveolar P_{CO_2} of about 40 mm. on the average, whereas the hypoxic altitude dweller has a much lower P_{CO_2}. Consequently, the average congenital cyanotic individual appears to be in a state of metabolic acidosis which is only partially compensated by an increased ventilation. Such a tendency toward metabolic acidosis in congenitally cyanotic individuals has been reported previously by Morse and Cassells (12).

The acid-base picture in chronic altitude hypoxia is, by contrast, one of compensated respiratory alkalosis. This situation enables the mountain dweller to load his arterial blood at a relatively high P_{O_2} and at the same time to maintain a normal pH.

Although the mechanisms and the sequence of events involved in the acid-base adjustments to altitude hypoxia seem reasonably well understood, the same cannot be said for the individual who is hypoxic from congenital heart disease. It is not clear, for example, why the respiratory center does not respond sufficiently to relieve the acidosis that tends to be present in these individuals. Regardless of its origin it may be that an acidosis is of advantage to the congenital cyanotic because it would tend to aid in the unloading of oxygen in the tissues without hindering to a comparable degree the loading of oxygen in the lungs. Reference to the nomograms of Dill, Talbott, Consolazio, and Edwards (11, 13) will make this clear.

Consider an individual whose arterial saturation is 65 per cent because of the presence of a right to left shunt. The blood leaving his lungs will be almost completely saturated (say 97 per cent) whatever the pH, since the Bohr effect is very small at high saturations. If the pH of the mixed arterial blood beyond the shunt were 7.4 the P_{O_2}, at 65 per cent saturation, would be 34 mm. On the other hand if the pH were 7.25, the P_{O_2} at the same saturation would be 39 mm. The P_{O_2} of mixed venous blood leaving the tissue would be affected to about the same degree, and we may conclude that the presence of the acidosis resulted in delivery of O_2 to the tissues at a pressure about 5 mm. higher than would have occurred at a normal pH.

Thus it appears that the acidosis observed in some congenital cyanotic individuals may be considered as an advantageous adaptation insofar as delivery of O_2 to the tissues is concerned, although it may of course be disadvantageous in other regards.

One might ask whether acidosis would similarly be of benefit in altitude hypoxia. The answer seems to be that it would not, because in the hypoxia of high altitude the blood leaving the lungs is at a relatively low saturation and the Bohr effect would hinder the loading of O_2 in the lungs sufficiently to offset any advantage to unloading in the tissues.

This can be illustrated by a specific example again with reference to Dill, Talbott, and Consolazio's (11) nomogram. Consider an individual at altitude, who has an oxygen carrying capacity of 30 volumes per cent, who is ventilating at such a rate that his arterial P_{O_2} is 33 mm., and whose A-V oxygen difference is 5 volumes per cent. At pH 7.4 the arterial saturation will be 65 per cent and the mixed venous saturation 48 per cent corresponding to P_{O_2}'s of 33 mm. and 25 mm., respectively. At pH 7.25 the arterial saturation will be 56 per cent and the mixed venous saturation 40 per cent corresponding to P_{O_2}'s of 33 and 25 mm., respectively. Thus an acidosis is evidently of no advantage to the delivery of O_2 in the case of altitude hypoxia.

The increase in oxygen carrying capacity with chronic hypoxia

It is well known that individuals who are chronically hypoxic tend to develop a polycythemia with a concomitant increase in the oxygen carrying capacity of the blood. The mechanism by which the increased hemoglobin is brought about is still obscure, nor is there general agreement as to its importance in the overall picture of acclimatization (14).

Data showing the magnitude of the increase in oxygen carrying capacity of the blood in man residing at various altitudes have been summarized by Hurtado (15). In Figure 4 we have plotted these data to show the oxygen carrying capacity as a function of the per cent saturation of the arterial blood. It is evident that an excellent linear relationship exists, the equation for the straight line being

$$C\ max = 67.1 - 0.476\ Sa, \qquad (1)$$

where C max is the oxygen carrying capacity in volumes per cent and Sa is the per cent saturation of arterial blood.

The combined O_2 carried by the arterial blood is the product of capacity times saturation or in this case

$$CaO_2 = 67.1\ Sa - 0.476\ Sa^2. \qquad (2)$$

Values for combined oxygen have also been plotted on Figure 4 as well as a curve calculated

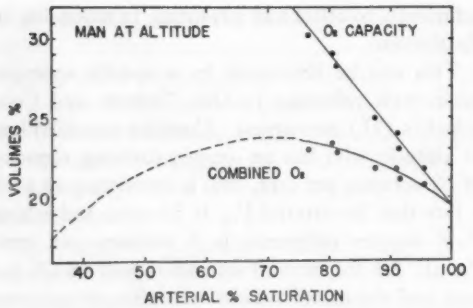

FIG. 4. COMBINED OXYGEN AND OXYGEN CARRYING CAPACITY AS A FUNCTION OF ARTERIAL SATURATION IN ALTITUDE DWELLERS

from Equation 2 and extrapolated to lower saturations than those for which data are available. Differentiation of Equation 2 with respect to Sa and setting the result equal to 0 shows that the combined oxygen has a maximal value at a saturation of 70.5 per cent.

This means that when man is chronically exposed to hypoxia the combined oxygen in his arterial blood will actually increase, because of the increased carrying capacity, until the hypoxia is so severe as to cause a saturation of 70 per cent. It is interesting in this connection that man does not live permanently at altitudes at which the arterial saturation lies below this value.

We have collected a number of our own measurements relating oxygen carrying capacity and per cent saturation of arterial blood which were made on 142 patients, many of whom were hypoxic because of a right to left circulatory shunt. Figures 5 and 6 show that the relationships between capacity and per cent saturation and combined oxygen and per cent saturation are somewhat similar to those observed for the altitude dweller.

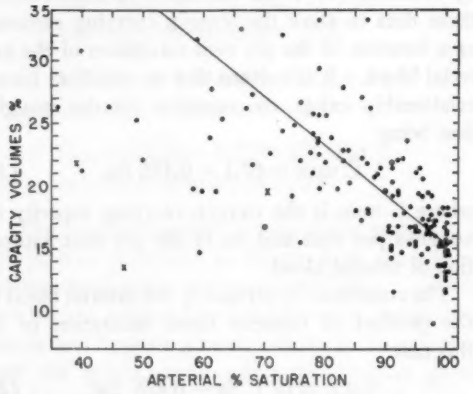

FIG. 5. OXYGEN CARRYING CAPACITY AS A FUNCTION OF ARTERIAL SATURATION IN PATIENTS STUDIED IN THIS INVESTIGATION

The straight line in Figure 5 was fitted to the points representing saturations of 75 per cent or above, and conforms to the equation

$$C\ max = 54.3 - 0.39\ Sa. \qquad (3)$$

The curved line in Figure 6 was derived from the straight line in Figure 5 and is represented by the equation

$$CaO_2 = 54.28\ Sa - 0.39\ Sa^2. \qquad (4)$$

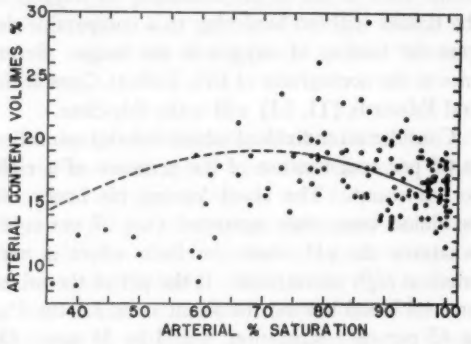

FIG. 6. COMBINED OXYGEN IN THE ARTERIAL BLOOD OF PATIENTS STUDIED IN THIS INVESTIGATION

This curve passes through a maximum at a saturation of 69.6 per cent.

In Figure 5 it is interesting that the points representing saturations less than 70 per cent fall for the most part below the straight line. This suggests the possibility that hypoxia more severe than that represented by a saturation of 70 per cent fails to stimulate any further increase in hemoglobin.

The importance of the increased O_2 combining power of the blood to the delivery of O_2 to the tissues in chronic hypoxia

The mountain dweller gains from an increased oxygen capacity because removal of a given amount of oxygen from the blood by the tissues produces less of a drop in the saturation and consequently the venous P_{O_2} will be higher than it

would be otherwise. The arterial P_{O_2}, since it is determined almost solely by the ventilation, will not be affected by an increased oxygen combining power of the blood.

The individual with shunt hypoxia will gain even more than the altitude dweller from an increased oxygen capacity, because in his case both arterial and venous P_{O_2}'s will be elevated. The following example will make this clear.

First consider a normal individual who is exposed to an altitude such that his arterial saturation is 75 per cent, whose oxygen carrying capacity is 20 volumes per cent, and whose oxygen consumption and cardiac output are such that his arterio-venous oxygen difference is 5 volumes per cent. His mixed venous saturation in this case will be 50 per cent. By reference to the O_2 dissociation curve and application of the Barcroft formula [4] (16) we can estimate his arterial, mixed venous, and mean tissue capillary P_{O_2}'s to be 40, 27 and 31 mm. Hg, respectively.

Now let this same individual increase his O_2 capacity to 30 volumes per cent leaving his ventilation, O_2 consumption and cardiac output as before. His arterial saturation will remain 75 per cent, but the content will have increased from 15 to 22.5 volumes per cent. The mixed venous content will be $22.5 - 5 = 17.5$ volumes per cent. The mixed venous saturation will be $17.5/30 =$ 58 per cent. The arterial, mixed venous and mean tissue capillary P_{O_2}'s will be 40, 31 and 34 mm., respectively. Thus the increased oxygen capacity has increased the mean capillary P_{O_2} by only 3 mm. Hg.

Let us now consider an individual at sea level with an O_2 capacity of 20 volumes per cent but with a right to left circulatory shunt such that his arterial blood is composed of a mixture of equal parts of blood that has passed through the lungs and of mixed venous blood. Let his cardiac output and oxygen consumption be such that his arterial-venous difference is 5 volumes per cent. We can consider the blood that has passed through his lungs as essentially 100 per cent saturated and to have an oxygen content of 20 volumes per cent. We can write two simultaneous equations as follows:

$$C_{a}o_{2} - C_{v}o_{2} = 5 \text{ vols. } \%,$$

$$C_{a}o_{2} = \frac{C_{v}o_{2} + 20 \text{ vols. } \%}{2}.$$

Solving those equations we find that arterial content is 15 volumes per cent and mixed venous content is 10 volumes per cent. The corresponding saturations are 75 per cent and 50 per cent, the same as in the case of the normal individual at altitude, and the arterial, mixed venous and mean capillary P_{O_2}'s will likewise be identical with those of the individual at altitude.

Now let us consider the above individual with the same cardiac output, O_2 consumption and degree of venous admixture but with his O_2 capacity increased to 30 volumes per cent. We can write the following simultaneous equations:

$$C_{a}o_{2} - C_{v}o_{2} = 5 \text{ vols. } \%,$$

$$C_{a}o_{2} = \frac{C_{v}o_{2} + 30 \text{ vols. } \%}{2}.$$

Solution of these equations and conversion of contents to saturations give us a saturation of 83 per cent for the arterial and 67 per cent for the mixed venous blood and P_{O_2}'s of 49, 35, and 40 mm. Hg for arterial, mixed venous and mean tissue capillary P_{O_2}. Thus the increased O_2 capacity has raised the mean capillary P_{O_2} from 31 to 40 mm., a gain of 9 mm., as compared with a gain of 3 mm. for the individual at altitude.

Resting O_2 consumption in shunt hypoxia

It would undoubtedly be a useful adaptation to a state of chronic hypoxia if the body could find a means of carrying on its basal activities with a diminished consumption of oxygen. That such an adaptation may actually occur was suggested by Bing, Vandam, Handelsman, Campbell, Spencer, and Griswold (1) who reported that in 28 of 30 patients who were chronically hypoxic from congenital cyanotic heart disease (Tetralogy of Fallot) the basal metabolism was either significantly below or at the lower limits of normal. Values for the basal metabolic rate as low as -48 were recorded and the average for the group was -23.

[4] Barcroft's (16) equation is as follows: Mean tissue capillary P_{O_2} = Venous P_{O_2} + $\frac{\text{Arterial } P_{O_2} - \text{Venous } P_{O_2}}{3}$.

In contrast to these striking results normal or slightly elevated values for basal oxygen consumption in congenital cyanotic heart disease have been obtained by Holling and Zak (17), Burchell, Taylor, Knutson, and Wood (18), Ernsting and Shephard (2) and Davison, Armitage, and Arnott (4). Thus the majority of the evidence indicates that no diminution in basal oxygen requirement occurs in chronic hypoxia resulting from congenital cyanotic heart disease, but the question is of such fundamental importance that we decided to satisfy our curiosity by measurements of our own.

None of the above mentioned investigators made measurements of oxygen consumption on a control group of acyanotic individuals but used "standard values" for comparison. Consequently, for our own investigation we decided to include a suitable control series.

Our chronically hypoxic series consisted of 42 individuals with cyanotic congenital heart disease. The arterial oxygen saturation in this group ranged from 61 per cent to 93 per cent and averaged 83 per cent. Our control series was composed of 45 individuals with non-cyanotic congenital heart disease, and arterial oxygen saturations of 94 per cent or higher. Various age groups were represented in both series. The measurements of oxygen consumption were made in connection with the procedure of cardiac catheterization which was being performed for diagnostic purposes. The subjects, who were in a fasting state, had received shortly before the procedure premedication consisting of morphine sulfate, 1 mg. per 5 Kg. body weight and scopalamine, 0.05 to 0.1 mg. per 5 Kg. body weight, and in the age groups up to 15 years Nembutal® suppositories, 2 mg. per Kg. body weight. The patients over 15 years usually received Nembutal® orally in a dose of 0.1 gm. Oxygen consumption measurements were based on the analysis of expired gas collected in a 100-liter recording spirometer. After two or three preliminary wash-out periods, the final collection was made during a 3-minute period. Analysis of the expired gas was performed on the Scholander (19) apparatus. Body surface area was estimated from height and weight according to Dubois (20). Oxygen saturation was determined by a modification of the Nahas (21) technique on blood samples drawn by arterial puncture.

RESULTS

Since it is well known that the resting oxygen consumption per square meter of surface area varies considerably with age, allowance for this was made in the interpretation of our data. The subjects in each series (acyanotic and cyanotic) were divided into five age groups and the mean oxygen consumption was determined for each group. The results are represented graphically in Figure 7. Data from Robinson (22) for the resting oxygen consumption of normal males of comparable ages are also shown for comparison.

It is clear that up to age 20 our data for both the cyanotic and acyanotic series agree remarkably well with those of Robinson. In the higher age groups, however, our values, while still showing no significant difference between cyanotic and acyanotic patients, deviate significantly from those of Robinson's. We are not certain as to the explanation for this discrepancy, but offer the suggestion that the older age groups were not as completely relaxed during the procedure partly perhaps because of less effective sedation and partly because of greater apprehension arising from more awareness of the potential hazards of cardiac catheterization.

At any rate, the important fact from the point of view of the present investigation is that there is no significant difference between the oxygen con-

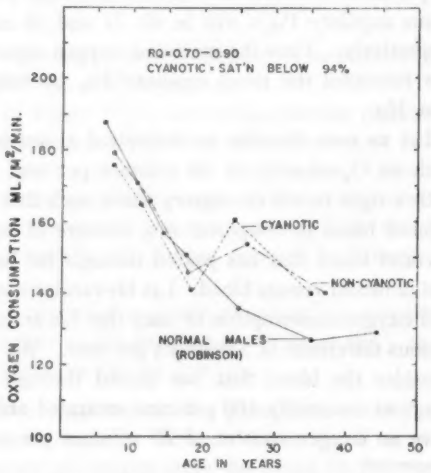

FIG. 7. RELATIONSHIP BETWEEN RESTING OXYGEN CONSUMPTION AND AGE IN CYANOTIC AND NON-CYANOTIC SUBJECTS

sumption of patients with chronic hypoxia arising from circulatory anomalies and that of those who had circulatory anomalies but no hypoxia. This is true whether the comparison is made between the two series as a whole or between similar age groups from the two series.

We have also compared our two series in another fashion on the basis of basal metabolic rates estimated by application of the Dubois (20) nomogram to our measured values for oxygen consumption. This comparison shows that, although in each series there is a slightly higher than normal B.M.R. value, the difference between the two series is not significant.

From this study we can only infer that the resting oxygen requirement is not depressed in our chronically hypoxic subjects and so are in agreement with the majority of other investigators mentioned in the introduction, and at variance with the findings of Bing, Vandam, Handelsman, Campbell, Spencer, and Griswold (1). Whether the low values for oxygen consumption obtained by Bing mean that his hypoxic subjects were vastly different from those studied by others or whether they were due to some systematic difference in his technique of measurement is a question that cannot be answered. Only one of our subjects had a B.M.R. as low as the average of Bing's group. Possibly Bing's subjects were more heavily sedated during the test, but no mention of sedation is made in his published reports. The objection might be raised that our cyanotic subjects were, on the average, less hypoxic than were Bing's, and that one therefore might expect less of a depression of metabolism in our group. This argument appears weak, however, in view of the fact that his data show no significant correlation between degree of hypoxia (as estimated by arterial saturation) and depression of the basal metabolic rate. It is unfortunate that Bing's study did not include measurements on a control group of noncyanotic subjects.

COMMENT

We have examined only a few of the many bodily adjustments that may occur as a result of exposure to chronic hypoxia, and have evaluated these on the basis of a single criterion, their effectiveness in elevating the P_{O_2} of the body. Other adjustments may be of equal or even greater importance and other criteria for their evaluation may prove more useful (23).

We have deliberately omitted any consideration of cardiac output because, in the case of shunt hypoxia, there is not sufficient information available as to how an alteration in cardiac output is distributed between systemic and pulmonary flows when a shunt is present (4, 18).

Adaptations are, by their nature, compromises in that concessions must be made in return for the advantages gained. For example, there is evidence that polycythemia is a predisposing factor in the formation of pulmonary and cerebral thrombi (24, 25). The importance and probability of occurrence of such undesirable effects must of course be taken into account in any complete evaluation of an adaptive mechanism, especially when individual cases are concerned.

SUMMARY

Although an increased pulmonary ventilation is an important adaptation to altitude hypoxia, it is of negligible value in raising the P_{O_2} of the body in shunt hypoxia. It may be of importance in the optimal regulation of acid-base balance in the latter instance however. In chronic altitude hypoxia the usual acid-base balance is one of compensated respiratory alkalosis. In shunt hypoxia there is a tendency for metabolic acidosis to be present. This may be of advantage in aiding the unloading of oxygen from blood to tissues. Polycythemia, which is usually present in both altitude and shunt hypoxia, is a more effective adaptation in the latter type because here it can raise the arterial as well as the mixed venous P_{O_2}. The basal oxygen requirement of individuals with shunt hypoxia does not appear to be lower than the normal.

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GASTROINTESTINAL WATER AND ELECTROLYTES. III. THE EQUILIBRATION OF RADIOBROMIDE IN GASTROINTESTINAL CONTENTS AND THE PROPORTION OF EXCHANGEABLE CHLORIDE (Cl_e) IN THE GASTROINTESTINAL TRACT [1]

By N. J. SWEET, J. NADELL,[2] AND I. S. EDELMAN [3]

(From the Department of Medicine, University of California School of Medicine, and the San Francisco Hospital, San Francisco, Calif.)

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Although chloride is distributed chiefly in the extracellular fluid, it is also found in intracellular water in as yet poorly defined quantities. The extracellular distribution includes plasma, free interstitial fluid, the interstitial fluid of dense connective tissue and bone, and transcellular fluid. The latter has been proposed as that portion of extracellular fluid formed, at least in part, as a result of active cellular transport mechanisms (1). The chemical composition of gastrointestinal contents differs in many ways from a simple ultrafiltrate of plasma. There is good evidence that chloride flux across the gastrointestinal mucosa, either directly or indirectly, involves active transport mechanisms (2, 3).

The physiologic significance of the chloride in transcellular fluid depends on its quantity and on the extent and rate of its equilibration with total exchangeable chloride, as well as other factors. The large volume of chloride-containing fluid in the gastrointestinal tract (4, 5) is one reason for the quantitative and functional definition of this moiety in relation to total body chloride.

Radiobromide (Br^{82}) was used as the tracer material in this study because of some disadvantageous physical characteristics of chloride[36] and chloride[38]. The half-life of Cl^{36} is 4×10^5 years (6), making it a potential hazard in terms of laboratory contamination and disposal. Cl^{38} has a half-life of only 37 minutes, which limits its value in studies requiring an equilibration period of more than a few hours (6).

The bromide: chloride ratio has been shown to be the same in tissues as in plasma after distribution equilibrium in animals, except for brain and cerebrospinal fluid (7–9). On the basis of these observations stable and isotopic bromide has been used extensively for *in vivo* estimations of total exchangeable chloride (Cl_e) (7, 10–15).

This communication presents observation on a) the fraction of total exchangeable chloride (Cl_e) in the lumen of the gastrointestinal tract of normal rabbits, b) the exchangeability of this chloride fraction based on bromide partition, and c) the amount of chloride in the gastrointestinal tract of man at post-mortem examination.

METHODS

A. Rabbits

Thirty-eight adult albino rabbits were studied in pairs, consisting of a male and a non-gravid female. The animals were allowed *ad libitum* ingestion of water, but food was withheld from the time of isotope injection until sacrifice. The fasting periods varied from 21 to 65 hours.

Each animal was injected intraperitoneally with 15 to 25 microcuries of KBr^{82} from calibrated syringes.[4] The injected material was made up as a neutral, sterile isotonic solution with saline. Observations on half-lives of decay on aliquots of the injected material fell within the reported values for Br^{82} (6), indicating that any small quantities of K^{42} present were not contributing significantly to radioassay.

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[2] Research Fellow of the National Heart Institute, U. S. Public Health Service.

[3] Established Investigator of the American Heart Association.

[4] KBr^{82} was supplied by the Oak Ridge National Laboratory of the Atomic Energy Commission. On the day of shipment each unit contained 5 millicuries of K^{42} and 120 millicuries of Br^{82}. Five half-lives for K^{42} were allowed to elapse prior to use, insuring negligible contamination. Br^{82} is a β^-, γ emitter with a half-life of 35.7 hours (6).

The techniques of collection of blood, bladder urine, stool and gastrointestinal contents were described in previous communications (1, 16). Urine and stool passed during the period of isotope equilibration were collected quantitatively in metabolism cages. In eight instances erythrocytes were aspirated from below the plasma layer of centrifuged heparinized blood obtained by cardiac puncture. The erythrocytes were hemolyzed in four times their volume of distilled water, and the hemolysates were filtered through double thickness paper. No attempt was made to remove trapped plasma, since the error due to plasma contamination in the specific activity of erythrocyte chloride would not exceed 10 per cent. Excreted stool and fecal contents of the distal portion of the large bowel were homogenized in a Waring blendor with measured volumes of distilled water, and filtrates collected after passage through glass-wool. Aliquots of plasma, lysed erythrocytes, bladder urine, cage urine, excreted stool, stool in the descending colon and gastrointestinal contents were taken for assay of radioactivity and chemical analysis. No attempt was made to prevent volatilization of HBr by neutralization of gastric contents during the brief exposure to room air.

One-ml. aliquots of all samples were plated on filter paper in metal planchets in triplicate, dried under an infrared lamp and covered with parafilm. Standards of three separate dilutions were prepared from aliquots of each of the injected solutions. Triplicate one-ml. plates were made from each dilution as above. It was found that addition of one drop of concentrated detergent solution to the standards and one or two drops of 50 per cent sucrose solution to all planchets improved the reproducibility of radioassay.

Assay of radioactivity was carried out with an end-window, thallium-activated sodium-iodide scintillation counter. Coincidence and self-absorption losses were found to be negligible. Consequently, these corrections were not applied. Corrections for background radiation and decay were applied to each assay.

Serum, urine, stool and gastrointestinal contents were analyzed for chloride content in triplicate by the Wilson and Ball method (17). Iced containers were used to sharpen the end-points.

B. Human subjects

Thirteen human subjects were studied at the time of post-mortem examination. The age, sex, weight and height of each subject was recorded. The criteria for patient selection and the post-mortem pathologic findings were described in the first report in this series (1). Direct collections were achieved by emptying each segment of the gastrointestinal tract. The details of collection and processing of these samples have been reported previously (1). A thin layer of mucus invariably clung to the mucosal wall of each segment. Quantitative emptying of each segment was not attempted to avoid contamination of the samples with gastrointestinal epithelium or blood.

Triplicate chloride analyses were carried out on aliquots of the diluted contents collected from each segment of the gastrointestinal tract by the Wilson and Ball method (17).

CALCULATIONS

A. Rabbits

Bromide is, for the most part, partitioned in direct proportion to chloride in body fluids (7–9). The application of radiobromide (Br^{82}) dilution to the measurement of total exchangeable body chloride content is justified on the same grounds as the use of bromide ($Br^{79/81}$) for this purpose, and yields results which closely approximate those obtained with radiochloride ($Cl^{36/38}$) (7, 14, 15).

The total exchangeable body chloride content (Cl_e) was calculated with the conventional dilution formula (1), corrected for urinary losses. Cardiac blood samples were drawn 21 to 65 hours after injection of Br^{82}. Urinary loss of Br^{82} was extremely variable in spite of the fact that the animals were matched for age, sex and body weight and kept on identical diets prior to fasting. In 14 rabbits fasted for 21 to 27 hours the cumulative urinary Br^{82} excretion rate averaged 8.1 per cent of the injected amount per day, with a range of 1.0 to 15.2 per cent per day; in 17 rabbits fasted for 41 to 48 hours the cumulative urinary Br^{82} excretion rate averaged 9.4 per cent of the injected amount per day, with a range of 5.1 to 22.2 per cent per day, while the two rabbits subjected to 64- and 65-hour fasts excreted Br^{82} at a rate of 4.7 and 3.6 per cent of the injected amount per day, respectively.

The partition of Br^{82} in proportion to chloride in gastrointestinal contents and erythrocytes was evaluated by relating the Br^{82}: chloride concentration of these samples to the Br^{82}: chloride concentration in serum and expressed as the specific activity ratio (S.A.R.). The S.A.R. is indicative of the fractional exchange of the bromide tracer relative to chloride, and exchange equilibrium is assumed to be complete when a value of 1.00 is obtained consistently (1, 18).

B. Human subjects

The intraluminal gastrointestinal chloride content has been expressed as an absolute quantity (mEq.), as the amount per unit of body weight (mEq. per kgm.) and as a percentage of the predicted total exchangeable chloride content (per cent of Cl_e) (cf. Table IX). The total exchangeable chloride content was predicted from previously published normal values (11, 12, 19). The available data on Cl_e in normal human subjects are too few in number to permit predictions corrected for age or body habitus. We have, therefore, applied single standards, taking into account only sex and body weight. It was assumed that the Cl_e was 29.0 mEq. per kgm. and 30.9 mEq. per kgm. of body weight in female and male subjects, respectively. There is abundant evidence from chloride balance data in humans to indicate that these predictions probably do not involve errors in excess of a factor of two. The need to establish some estimate of gastrointestinal chloride pool-size in terms of total amount of chloride in the body in humans justifies these crude approximations.

TABLE I

Plasma versus erythrocyte specific activity in the rabbit 21 to 24 hours after injection of radiobromide *

Rabbits	Specific Activity of Plasma	Specific Activity of Erythrocytes	Specific Activity Erythrocytes / Specific Activity Plasma
	cts/min/mEq X 10^3	cts/min/mEq X 10^3	ratio
3	441.5	449.6	1.02
4	436.3	439.1	1.01
5	143.9	144.8	1.01
6	165.3	160.1	0.97
7	63.19	67.26	1.06
8	58.86	57.70	0.98
9	81.60	78.45	0.96
10	71.47	72.89	1.02
		Mean	= 1.00
		s.d.**	= ±0.03

* Specific activity refers to the activity of radiobromide per mEq. of chloride ion.

** s.d. = $\sqrt{\frac{\Sigma (x-\bar{x})^2}{n-1}}$

RESULTS

A. Rabbits

The partition of Br^{82} between plasma and erythrocytes was evaluated by determining the S.A.R. of these samples in 8 rabbits 21 to 24 hours after the injection of Br^{82} (cf. Table I). The mean S.A.R. of 1.00 ± 0.03 indicates that Br^{82} is distributed in direct proportion to chloride across the red cell membrane. These data corroborate previous observations on bromide penetration into red cells (8, 14).

Sex-linked differences in body composition have been noted in human subjects in previous studies (10–12, 20). Sixteen male-female pairs were studied to evaluate the possibility of sex-linked differences in either Cl_e or gut chloride content. Each pair was matched for weight and age and subjected to identical periods of fasting and isotope equilibration. Although the Cl_e content of the

TABLE II

The exchangeable chloride (Cl_e) and gastrointestinal chloride content of male versus female rabbits *

	Male	Female	t	P
Number	16	16		
Body weight in Kg. ± s.d.	2.032 ± 0.145	2.030 ± 0.277	0.003	>0.9
Cl_e in mEq. ± s.d.	72.3 ± 8.7	69.0 ± 7.5	1.15	>0.2
Cl_e/body weight in mEq./Kg. ± s.d.	35.5 ± 3.2	34.2 ± 3.5	1.19	>0.2
"Total" G-I chloride as % of Cl_e ± s.d.**	17.6 ± 5.4	17.7 ± 5.4	0.052	>0.9

* Each pair of animals was matched for weight and studied after identical equilibration and fasting periods. Equilibration periods varied from 21 to 65 hours.

** "Total" G-I Cl_e refers to the intraluminal chloride content of the gastrointestinal tract from the cardia of the stomach to the mid-transverse colon.

TABLE III

The effect of varying equilibration periods of 21 to 65 hours on the estimated total exchangeable chloride (Cl_e) content in rabbits

Equilibration Period Hours	Number of Animals	Cl_e/Body Weight mEq./Kg. (mean ± s.d.)	t	P
21 - 24	14	33.8 ± 2.92	1.74	>0.10
40 - 48	17	35.7 ± 3.14		
64 - 65	2	36.3*		

* The two values averaged are 35.9 and 36.7 mEq./Kg. of body weight. Both values fall within one standard deviation of the mean value after either 21 - 24 or 40 - 48 hours of equilibration.

male (35.5 ± 3.2 mEq. per kgm. of body weight) is slightly higher than that of the female (34.2 ± 3.5 mEq. per kgm. of body weight), neither the differences in Cl_e nor in the "total" gastrointestinal chloride achieves statistical significance (cf. Table II). In view of these findings subsequent calculations were made without regard to the sex of the individual animals.

To evaluate the possibility of slow penetration of bromide into tissues beyond the accepted 24 hours required for distribution equilibrium, studies on the effect of varying equilibration periods from 21 to 65 hours on the estimated Cl_e were carried out in 33 rabbits. These data are summarized in Table III. Although the Cl_e per kgm. of body weight is slightly higher in the group where 40 to 48 hours were allowed for equilibration, this difference, 1.9 mEq. of chloride per kgm., is not statistically significant ($p > 0.10$). These data confirm and extend those reported on the equilibration of bromide and radiochloride (8, 9, 21).

The data tabulated in Tables II and III indicate that sex or prolongation of the period of isotope equilibration may not influence significantly the Cl_e per kgm. of body weight. The data on these groups have been combined, and the serum chloride and Cl_e are listed in Table IV. The serum chloride averaged 98.4 ± 6.4 mEq. per liter and the Cl_e averaged 71.0 ± 8.7 mEq., or 34.9 ± 3.5 mEq. per kgm. of body weight. Weir (7) reported a mean Cl_e of 30.2 mEq. per kgm. of body weight in 10 rabbits estimated by bromide dilution after 1 to 1.5 hours of equilibration, which indicates that 80 to 90 per cent of distribution equilibrium is reached in the first 1 to 2 hours.

The penetration of Br^{82} into the gastrointestinal tract has been evaluated by measurements of the

TABLE IV

*The exchangeable chloride (Cl_e) content in the rabbit**

	Body Weight Kg.	Serum Chloride mEq./L	Exchangeable Chloride mEq.	Exchangeable Chloride mEq./Kg.
Mean	2.038	98.4	71.0	34.9
s.d.	±0.221	±6.4	±8.7	±3.5
Coefficient of variation	10.8%	6.5%	8.2%	10.0%
Number of animals	33	33	33	

* Equilibration period 21 - 65 hours.

TABLE V

The equilibration of Br^{82} with intraluminal gastrointestinal chloride in the rabbit

	No. of Animals	Equilibration Period 21-24 hours	No. of Animals	Equilibration Period 41-48 hours	t	P
Stomach S.A.R. (mean ± s.d.)*	6	0.86 ± 0.03	19	0.90 ± 0.02	2.96	<0.01
Small intestine S.A.R. (mean ± s.d.)*	6	0.92 ± 0.05	19	1.00 ± 0.04	3.65	<0.01
Cecum & proximal half of transverse colon S.A.R. (mean ± s.d.)*	6	0.95 ± 0.04	19	0.94 ± 0.06	0	1.00
"Total" gastrointestinal contents** S.A.R. (mean ± s.d.)	14	0.94 ± 0.06	19	0.94 ± 0.03	0	1.00

* S.A.R. = specific activity ratio.

** "Total" gastrointestinal contents refers to the intraluminal chloride content from the cardia of the stomach to the mid-transverse colon. The gastrointestinal contents were pooled and analyzed as a single sample in 8 animals after 21-24 hours of equilibration.

S.A.R. of gastric, small bowel and proximal large bowel contents after 21 to 24 hours and 41 to 48 hours of equilibration. These data are listed in Table V. Equilibration is almost complete in 24 hours, but there appears to be some increase in equilibration during the second day in both gastric and small bowel contents ($p < 0.01$). The S.A.R. for stomach may seem to be somewhat less than 100 per cent equilibrated at 48 hours because of volatilization of small amounts of HBr at the time of sample collection. Gamble, Robertson, Hannigan, Foster, and Farr (15) noted more rapid penetration of radiobromide compared with radiochloride into gastric juice of man during the first 2 hours of equilibration. Their data cannot be directly compared with ours because of differences in species and time of sampling. Proportional distribution of bromide to chloride between serum and gastric juice has, however, been found in patients after chronic bromide ingestion (22).

Data on the effect of short fasting periods on the quantity of intraluminal gastrointestinal chloride relative to Cl_e have been summarized in Table VI. There appears to be some decline in intraluminal chloride content during the second fasting day, *i.e.*, total gut chloride of 20.0 ± 5.6 per cent of Cl_e after 24 hours of fasting versus a value of 16.0 ± 4.5 per cent of Cl_e after 48 hours of fasting ($0.05 > p > 0.02$). To be certain of this effect would require more prolonged periods of observation. Since there may also be some small gain in distribution equilibrium during the second 24 hours of fasting, a 48-hour equilibration and fasting period was used as the basis for measuring intraluminal chloride content.

The amount and distribution of intraluminal chloride after 48-hour equilibration and fasting periods are summarized in Table VII. The "total" gut chloride is quite significant in quantity, averaging 16.0 ± 4.5 per cent of Cl_e. Gastric

TABLE VI

*The effect of short fasting periods on intraluminal gastrointestinal chloride content**

	Fasting Periods		t	P
	21 - 27 hours	41 - 48 hours		
"Total" G-I chloride % Cl_e (mean ± s.d.)	20.0 ± 5.6	16.0 ± 4.5	2.16	<0.05 >0.02
Number of animals	14	17		

* Isotope equilibration period was the same as the fasting period in each case.

TABLE VII

Intraluminal gastrointestinal chloride content in the rabbit *

	Stomach		Small Intestine		Cecum & Proximal Transverse Colon		"Total" G-I	
	mEq.	% of Cl_e	mEq.	% of Cl_e	mEq.	% of Cl_e	mEq.	% of Cl_e
Mean	8.7	11.7	1.8	2.5	1.2	1.7	11.8	16.0
s.d.	±3.4	±4.4	±0.4	±0.7	±0.3	±0.4	±3.6	±4.5
Coefficient of variation	39%	38%	22%	28%	25%	24%	31%	28%
Number of animals	17		17		17		17	

* Equilibration and fasting intervals of 41-48 hours, water allowed *ad libitum*.

chloride provides the bulk of this quantity, with a mean of 11.7 ± 4.4 per cent of the Cl_e, which is equivalent to 73 per cent of the total gastrointestinal chloride content. This is in direct contrast to the distribution of gut sodium and potassium in rabbits, where 72 per cent of gut sodium and 63 per cent of gut potassium are in the cecum and proximal half of the transverse colon (1, 16). The small amount of chloride in the proximal segment of the large bowel, 1.7 ± 0.4 per cent of the Cl_e, suggests efficient cecal conservation of chloride. This is borne out by the studies on distal large bowel chloride and the small daily fecal losses of chloride.

Table VIII summarizes the data on the intraluminal chloride content of the distal colon and the rates of stool chloride excretion during 24- to 48-hour fasting periods. The chloride content of the distal segment of the large bowel is minute, and the fecal chloride excretion rate is quite low, averaging about 1.0 per cent of the Cl_e per day. Although chloride exchange between plasma and gut apparently proceeds along the full length of the gastrointestinal tract, it would appear that net flow is in the intraluminal direction at the oral end and in the direction of the blood stream at the aboral end of the gut.

B. Human subjects

The results obtained on post-mortem examination of gut chloride in man are enumerated in Table IX. The interval between demise and collection of samples varied from 6 to 22 hours. Inspection of these data indicates that there is no correlation between gut chloride content and the post-mortem interval. Gastric chloride averaged 14.7 mEq., or 0.9 per cent of the predicted Cl_e, which is approximately 50 per cent of "total" gut chloride (31.7 mEq., or 1.9 per cent of the predicted Cl_e). Most of the remainder was found in the small bowel (11.4 mEq., or 0.7 per cent of the Cl_e). These data, although indicating significantly smaller gut chloride contents in man as compared to rabbits, reveal a similar pattern of

TABLE VIII

The intraluminal chloride content of the distal colon and the rate of stool chloride excretion in rabbits

	Distal Colon and Rectum			Stool Chloride per 24 Hours*		
	mEq.	% "Total" G-I Chloride	% Cl_e	mEq.	% "Total" G-I Chloride	% Cl_e
Mean	0.06	0.5	0.09	0.73	6.2	1.03
Range	0.02 - 0.13	0.2 - 1.1	0.04 - 0.16	0 - 2.64	0 - 22.4	0 - 3.72
Number of animals	13			13		

* Stool collections were made over a 1 to 2 day period and expressed as chloride excreted per day. All animals were fasting during the collection periods.

chloride distribution along the length of the gastrointestinal tract. It must be emphasized, however, that these data are not reliable, since agonal or post-mortem changes in intraluminal chloride content may have occurred. Species differences for intraluminal water content have been reported (23). Definitive measurements in man require access to gut contents immediately after sudden death in previously well individuals.

DISCUSSION

Total body chloride estimated *in vivo* in man by isotope dilution averages 31 mEq. per kgm. of body weight in adult males and 29 mEq. per kgm. of body weight in adult females (11, 12, 19). Infants have significantly more chloride, averaging 51 mEq. per kgm., which is to be expected in view of their higher body sodium and water contents (13, 24, 25). Weir (7) estimated the Cl_e in rabbits to be 30 mEq. per kgm. based on 1- to 1.5-hour bromide dilution. This figure is about 85 per cent of the values of 35.5 mEq. per kgm. and 34.2 mEq. per kgm. for male and female rabbits, respectively, with 21 or more hours of equilibration of Br^{82} (cf. Table II).

The distribution of body chloride is important in the interpretation of both metabolic balance data and the movements of water and ions across cell membranes. Chloride in the lumen of the gastrointestinal tract is obviously extracellular. In man, the concentration of chloride decreases and the concentration of bicarbonate increases progressively from stomach to colon (5, 26, 27). The chloride concentration varies from about 150 mEq. per liter in the stomach to 50 to 80 mEq. per liter in the colon. There is a similar pattern of intraluminal chloride distribution in the fasting rabbit and in man studied post mortem. The blood-to-gut partition of Br^{82} parallels chloride distribution closely, as evidenced by the S.A.R. of 0.90, 1.00 and 0.94 for stomach, small intestine and proximal large bowel contents, respectively (cf. Table V). These data support the thesis that intraluminal chloride is an integral part of the body chloride pool.

Direct evidence for bidirectional flow of chloride has been obtained by Hogben (3) for the gastric mucosa of the frog and by Visscher and his associates (28–31) for the small bowel mucosa of the dog. Gastric transport of chloride is energy dependent and oriented from serosa to mucosa (3). Intestinal transport of chloride probably has an active component as well. Bidirectional flux is highest in the jejunum and lowest in the colon (28–31). At the aboral end of the bowel, chloride movement is oriented from gut to blood. Isotonic chloride solutions placed in the ileum or proximal colon show consistent diminution in chloride concentration and a reciprocal rise in bicarbonate concentration, while the sum of the concentrations of these anions remains unchanged (27, 32, 33). Taken together, these observations justify the identification of intraluminal chloride as a distinct subdivision of total extracellular chloride.

The rabbit has an impressive amount of intraluminal chloride. After 48 hours of fasting, 16 per cent of the Cl_e is in the gut and 73 per cent of this quantity, or 11.7 per cent of the Cl_e, is in the stomach (cf. Table VII). Since chloride in the proximal colon is only about 1.5 to 2.0 per cent of the Cl_e and the fecal excretion rate is only about 1.0 per cent of the Cl_e per day under fasting conditions, intestinal conservation of chloride is clearly an efficient process.

Using multiple simultaneous dilution techniques it has been estimated that total intracellular chloride is 30 to 40 per cent of the Cl_e (11, 12, 19). Since the methods for estimating extracellular fluid exclude gut contents, intraluminal chloride is mistakenly included in these intracellular figures. In the rabbit, Cl_e averages 35 mEq. per kgm.; assuming a plasma-interstitial compartment of 20 per cent of body weight (34) and a serum chloride concentration of 100 mEq. per liter, there would be 15 mEq. of chloride per kgm. of body weight outside of this phase. At least 30 per cent of this fraction, or 6 mEq. per kgm., is intraluminal, and less than 25 per cent of the Cl_e is intracellular. This does not take into consideration other transcellular fluids so that even this figure is too high.

Although post-mortem studies on distribution of electrolytes in man are unreliable, it is of interest to note the smaller quantities of intraluminal chloride compared to those in the rabbit, averaging approximately 2 per cent of the predicted Cl_e (cf. Table IX). The pattern of distribution along the length of the gastrointestinal tract is much the same as in rabbits, about 50 per cent of "total"

TABLE IX

Intraluminal gastrointestinal chloride content in the human studied post mortem

Sex	Pathological Diagnosis	Postmortem Interval	Age	Body Weight	Predicted Cl_e	Stomach			Small Intestine			Cecum and Proximal Transverse Colon			"Total" G-I		
		hours	yrs.	Kg.	mEq.	mEq.	mEq./Kg.	% of Cl_e	mEq.	mEq./Kg.	% of Cl_e	mEq.	mEq./Kg.	% of Cl_e	mEq.	mEq./Kg.	% of Cl_e
F-1	Recent myocardial infarction	6	77	35.0	1015	5.9	0.17	0.58	6.9	0.20	0.68	1.1	0.03	0.11	13.9	0.40	1.37
M-2	Rheumatic heart disease with mitral stenosis	11	53	51.8	1601	5.3	0.10	0.33	6.4	0.12	0.40	1.7	0.03	0.10	13.3	0.26	0.83
M-3	Hypertensive cardiovascular disease	22	51	43.6	1347	32.5	0.75	2.42	7.6	0.18	0.57	1.9	0.04	0.14	42.0	0.96	3.12
F-4	Pituitary tumor	9	54	58.2	1688	2.6	0.04	0.15	15.9	0.27	0.94	2.6	0.04	0.15	21.0	0.36	1.25
M-5	Squamous cell carcinoma of lung	7	64	43.6	1264	21.7	0.50	1.72	11.8	0.27	0.93	4.1	0.09	0.32	37.6	0.86	2.97
F-6	Cerebral arteriosclerosis with encephalomalacia	6	56	58.2	1688	14.6	0.25	0.87	5.5	0.10	0.33	10.4	0.18	0.62	30.5	0.53	1.81
F-7	Hypertensive and arteriosclerotic heart disease	22	68	63.4	1839	2.9	0.05	0.16	14.3	0.23	0.78	5.3	0.08	0.29	22.5	0.35	1.22
M-8	Paralysis agitans and bronchopneumonia	16	69	63.2	1953	2.7	0.04	0.14	15.0	0.24	0.77	8.1	0.13	0.41	25.8	0.41	1.32
F-9	Cerebral thrombosis	20	67	65.0	1855	38.4	0.59	2.04	8.7	0.13	0.46	0	0	0	47.1	0.72	2.50
M-10	Dissecting aneurysm of ascending aorta	19	59	68.0	2101	1.9	0.03	0.09	16.2	0.24	0.77	1.2	0.02	0.06	19.3	0.28	0.92
M-11	Recurrent myocardial infarction	16	72	64.5	1993	11.2	0.17	0.56	2.5	0.04	0.13	14.0	0.23	0.70	27.7	0.43	1.39
M-12	Chromophobe adenoma of pituitary	7	50	70.0	2163	24.2	0.35	1.12	21.9	0.31	1.01	7.7	0.11	0.35	53.8	0.77	2.49
M-13	Traumatic demyelinization of cervical spinal cord	12	76	54.5	1684	26.8	0.49	1.59	14.9	0.27	0.88	16.0	0.29	0.95	57.7	1.06	3.43
					Mean:	14.7	0.27	0.91	11.4	0.20	0.67	5.7	0.10	0.32	31.7	0.57	1.89
					Range:	1.9 - 38.4	0.03 - 0.75	0.09 - 2.42	2.5 - 21.9	0.04 - 0.31	0.13 - 1.01	0 - 16.0	0 - 0.29	0 - 0.95	13.3 - 57.7	0.26 - 1.06	0.83 - 3.43

gastrointestinal chloride in the stomach, 35 per cent in the small bowel, and 15 per cent in the proximal half of the large bowel. The validity of these observations is not yet established, however, for the previously stated reasons.

SUMMARY

Intraluminal gastrointestinal chloride content was measured in rabbits and in human subjects studied post mortem. In the former, gut chloride was referred to Cl_e estimated with KBr^{82}, while in the latter gut chloride was referred to the predicted Cl_e values.

Total exchangeable chloride averaged 34.9 ± 3.5 mEq. per kgm. of body weight in rabbits. Of this, 16.0 ± 4.5 per cent was in the lumen of the gastrointestinal tract, with 11.7 ± 4.4 per cent in the stomach, 2.5 ± 0.7 per cent in the small intestine, and 1.7 ± 0.4 per cent in the cecum and proximal half of the large intestine. Radiobromide exchange equilibrium was complete to within 10 per cent for all segments of the gastrointestinal tract 48 hours after injection. No significant difference in either the Cl_e or the quantity of intraluminal gastrointestinal chloride was found between male and female rabbits.

Human subjects at post-mortem examination had relatively small amounts of intraluminal gastrointestinal chloride; the mean values were 1.9 per cent of the predicted Cl_e in the "total" gastrointestinal tract, with 0.91 per cent in the stomach, 0.67 per cent in the small intestine, and 0.32 per cent in the cecum and proximal transverse colon. The quantity of intraluminal chloride in normal man cannot be reliably inferred from these data.

The implications of these data are discussed in terms of the dynamics of chloride transport across the gastrointestinal mucosa and the anatomy of body chloride.

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GASTROINTESTINAL WATER AND ELECTROLYTES. IV. THE EQUILIBRATION OF DEUTERIUM OXIDE (D_2O) IN GASTRO-INTESTINAL CONTENTS AND THE PROPORTION OF TOTAL BODY WATER (T.B.W.) IN THE GASTRO-INTESTINAL TRACT [1]

By F. GOTCH,[2] J. NADELL,[3] AND I. S. EDELMAN [4]

(From the Department of Medicine, University of California School of Medicine, and the San Francisco Hospital, San Francisco, Calif.)

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The concept of the anatomy of body water distribution as a two-compartment system consisting of intracellular and extracellular fluid has been shown to be inadequate (1–5). The heterogeneous nature of the extracellular fluid compartment has been established by previous studies on bone (2), dense connective tissue (6), and transcellular fluid (3–5). Cizek (3) has demonstrated that intraluminal gut water is a significant subdivision of body water in a number of species.

Neglecting the contribution of transcellular fluid to body water results in considerable errors in the derived normal values for body water compartments. Furthermore, transcellular fluid, if large enough in volume, must be considered as potentially important in determining the volume and osmolarity of plasma, interstitial and intracellular fluid by ion and water flux in or out of transcellular pools in response to metabolic stimuli.

In the preceding three papers of this series we reported the measured amount of sodium, potassium and chloride contained within the lumen of the gut in rabbits and in human subjects at post mortem (4, 5, 7). The present study is similar in design to these previous experiments and presents observations on a) the amount of intraluminal gastrointestinal water expressed as a fraction of total body water (T.B.W.) and the extent of deuterium oxide (D_2O) exchange equilibrium in gut contents in rabbits, and b) the amount of intraluminal water in man at post-mortem examination.

METHODS

A. Rabbits

Forty adult albino rabbits were studied in pairs consisting of a male and a non-gravid female. The animals were fasted and thirsted. All urine passed during the period of isotope equilibration was collected in a metabolism cage. This period varied from 2 to 5 hours. Each animal was injected intraperitoneally with 2 ml. of D_2O [5] from a calibrated syringe. At the end of the equilibration period each animal was anesthetized with 2 ml. of 2 per cent sodium pentobarbital injected into a dorsal ear vein and was then weighed to the nearest gram. A blood sample was obtained at this time by cardiac puncture through the intact chest wall with a syringe containing dry heparin. The syringe was capped and centrifuged immediately after collection, and the separated plasma was aspirated, sealed in a glass ampoule and stored in a freezer.

The gastrointestinal tract was removed in three segments by cutting between double ligatures placed at the cardia of the stomach, the pylorus, the ileocecal valve and at a position in the transverse colon where there was a transition point between semi-solid and solid stool pellets. After removal each segment was washed with distilled water, dried with towels and weighed to the nearest gram.

The contents of each segment were milked into one ligated end, a small incision was made, and an aliquot of contents was expressed into a dried test tube, which was quickly stoppered and centrifuged. The supernatant was then aspirated and sealed in a glass ampoule and stored in a freezer. Each segment was then opened longitudinally, and the remaining contents were evacuated into a clean container by gently stripping and then washing the mucosal surface with distilled water. The

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[2] Research Fellow of the American Heart Association.

[3] Research Fellow of the National Heart Institute of the United States Public Health Service.

[4] Established Investigator of the American Heart Association.

[5] Deuterium oxide, 99.6 per cent pure, was obtained from Abbott Laboratories as a sterile isotonic saline solution.

TABLE I

Effect of equilibration time on measured total body water

Number of rabbits			Equilibration time (*hours*)	Body weight mean ± s.d.* (*Kg.*)	Total body water as % body wt. mean ± s.d.*	t Value	p Value†
Male	Female	Total					
5	5	10	2	2.46 ± .27	71.0 ± 7.0		
4	5	9	3	2.05 ± .25	75.1 ± 5.5	1.44	>0.1
5	5	10	4	2.16 ± .23	74.4 ± 3.5	1.29	>0.2
5	5	10	5	2.20 ± .20	72.0 ± 4.4	0.39	>0.5

* s.d. $= \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$.

† Compared with 2-hour value for T.B.W. in each instance.

evacuated contents were quantitatively transferred with multiple distilled water rinses into a graduated cylinder to which the remainder of the previously centrifuged aliquot was added. The diluted contents were thoroughly mixed, the volume recorded and an aliquot taken for determination of solids. The wall of each segment was dried and reweighed.

Ten-ml. aliquots of the diluted contents were pipetted in duplicate into tared weighing bottles. The solid content of each sample was estimated gravimetrically after drying at 105° C for 72 hours. All duplicates checked within 10 per cent.

The concentration of D_2O in blood was determined in duplicate by the falling-drop method (8). The maximal acceptable difference between duplicates was 0.006 volume per cent. Only single analyses were carried out on urine samples since less than 1 per cent of the tracer was excreted during the equilibration periods. The concentration of D_2O in intraluminal water was determined by mass spectrometer analysis (9).[6] Each of these analyses was done in duplicate, and all duplicates checked within 0.006 atom per cent D.

B. Human subjects

The gastrointestinal tracts were removed and the contents analyzed in 13 human subjects at autopsy. The technique of sample collection was described previously (4). Water content was estimated as described for the rabbits. Subjects selected for study had no gastrointestinal disease and minimal clinical abnormality of fluid and electrolyte metabolism. The body weight and height of each subject were measured. The appendix to the first report in this series (4) describes the pertinent clinical and pathological findings in these cases.

CALCULATIONS

Rabbits

Total body water was calculated from the well-known isotope dilution formula (1). The amount of tracer retained was assumed to be the amount injected minus the amount excreted in the urine during the equilibration period. Isotope excretion varied between 0.1 per cent to 0.6 per cent of the injected D_2O. The maximum combined errors in this calculation are less than 5 per cent.

The volume of intraluminal water in each gut segment was calculated in the following manner:

a) *Weight of intraluminal contents* =
Weight intact gut segment − Weight of segment wall

b) *Total solids in intraluminal contents* =
(Volume diluted contents) (Weight solids in grams per ml.)

c) *Volume of intraluminal water* =
Weight of intraluminal contents − Total solids in intraluminal contents

Accumulated errors in the final calculation are 10 per cent or less.

Completeness of D_2O exchange between plasma water and intraluminal water was determined from the ratio of D_2O concentration of intraluminal water to plasma water. This ratio is designated the specific activity ratio (S.A.R.) (2, 4).

When exchange is complete, S.A.R. is unity since the concentration of D_2O will be the same in both intraluminal water and plasma water. The value of S.A.R. is assumed to represent the fraction of gut water exchanged with extracellular water (2, 4).

Human subjects

Total body water was estimated in these subjects from data derived from D_2O dilution studies on normal subjects in this age group (10). The predicted T.B.W. was calculated, using the values 22.2 liters per square meter in males and 17.1 liters per square meter in females.

Volume of intraluminal water was calculated by the methods outlined above for the rabbit.

RESULTS

Rabbits

Uniform dispersal of a tracer theoretically occurs after an infinite period of time (11). A prac-

[6] These analyses were performed by Mr. Brad Pearson under the direction of Dr. A. K. Solomon at the Biophysics Laboratory of the Harvard Medical School in Boston, for which we are most grateful.

tical means for judging equilibrium of distribution, however, is to determine the time required for constant volumes of dilution, in this case T.B.W. The effect of the time allowed for equilibration on the apparent T.B.W. is summarized in Table I. Mean T.B.W. values at 3, 4 and 5 hours of equilibration are compared to the 2-hour values. No significant differences were found among these groups, nor was there a trend in either direction. Two hours is probably an adequate period for D_2O equilibration in this species.

Data on T.B.W. and "total" intraluminal gastrointestinal water[7] in animals matched carefully for weight, age and the period of isotope equilibration and compared on the basis of sex are tabulated in Table II. The differences in T.B.W. and the gut water content of male and female rabbits were not statistically significant.

The absolute magnitude of intraluminal water content and its isotope exchange characteristics in each segment of the tract are given in Tables III and IV.

The mean volume of intraluminal water, expressed as per cent of T.B.W., in the stomach, small intestine and large intestine is 4.1, 2.0 and 6.0 per cent, respectively (cf. Table III). The sum of these, or "total" intraluminal water, is 12.1 per cent of T.B.W. The coefficients of variation for these data are large, varying between 22 and 50 per cent, and reflect the considerable variation in the volume of intraluminal gut water found in different animals in spite of rigidly controlled experimental conditions.

The specific activity ratios of gut water to plasma water at 2, 3, 4 and 5 hours' equilibration for stomach, small intestine and large intestine are listed in Table IV. The p values in each instance refer to comparison of the measured S.A.R. with unity, the theoretical value for complete equilibration. Equilibration is complete in the large bowel segment at 2 hours. In the small intestine, equilibration is nearly complete at 2 hours, with an S.A.R. of 0.95 ± 0.05, and is complete at 3 hours, with an S.A.R. of 0.99 ± 0.03. There is a significantly slower rate of exchange of water in the stomach. The S.A.R. of ± 0.86 at 2 hours differs significantly from 1.00 ($p < 0.001$). At 3 hours only 93 per cent of water in the stomach has exchanged with plasma water, but at 4 hours complete exchange is demonstrated (S.A.R. = 0.98 ± 0.05).

[7] "Total" intraluminal gastrointestinal water refers to water contained in the gut from the cardia of the stomach to the mid-transverse colon.

TABLE II

The total body water and "total" intraluminal gastrointestinal water of male versus female rabbits†*

	Male	Female	t Value	p Value
Number	13	13		
Body weight in Kg. ± s.d.	2.15 ± .18	2.14 ± .16		
T.B.W. as % body weight ± s.d.	73.3 ± 2.6	74.8 ± 3.5	1.25	>0.2
"Total" G.I. water as % T.B.W.	11.6 ± 1.9	12.5 ± 3.2	0.82	>0.4

* "Total" intraluminal gastrointestinal water refers to water contained in the gut from the cardia of the stomach to the mid-transverse colon.

† Each pair of rabbits was matched for weight, age and period of isotope equilibration.

TABLE III

The intraluminal gastrointestinal water content in the rabbit

	Stomach			Small intestine			Cecum and proximal transverse colon			"Total" G.I.		
	(*ml.*)	(*ml./Kg.*)	(% *T.B.W.*)	(*ml.*)	(*ml./Kg.*)	(% *T.B.W.*)	(*ml.*)	(*ml./Kg.*)	(% *T.B.W.*)	(*ml.*)	(*ml./Kg.*)	(% *T.B.W.*)
Mean	65	30	4.1	31	18	2.0	93	44	6.0	189	90	12.1
s.d.	±15	±7	±0.9	±12	±9	±1.0	±22	±13	±1.6	±40	±24	±2.7
Coefficient of variation	23%	23%	22%	39%	50%	50%	24%	30%	27%	21%	27%	22%
Number of rabbits*	29	29	28	29	29	28	27	27	26	26	26	25

* All animals were allowed 3 to 5 hours of isotope equilibration and thirsted 3 to 5 hours as summarized in Table I.

TABLE IV

The equilibration of D_2O between plasma and gastrointestinal contents

Equilibration time (*hours*)	Stomach				Small intestine				Cecum and transverse colon			
	No. of animals	S.A.R. mean ±s.d.	t*	p	No. of animals	S.A.R. mean ±s.d.	t*	p	No. of animals	S.A.R. mean ±s.d.	t*	p
2	10	0.86±.08	5.60	<0.001	9	0.95±.05	2.94	<0.02 >0.01	8	1.00±.09	0	1.0
3	9	0.93±.07	3.04	<0.02 >0.01	9	0.99±.03	1.00	>0.3	8	1.01±.04	.71	0.5
4	10	0.98±.05	1.25	>0.2	10	1.00±.03	0	1.0	10	1.01±.03	1.11	0.3
5	10	1.01±.02	1.67	>0.1	10	1.02±.03	2.22	>0.05 <0.10	10	1.01±.03	1.11	0.3

$$* t = \frac{1.00 - \bar{x}}{\frac{s.d.}{\sqrt{n}}}.$$

Human subjects

The volumes of measured intraluminal gastrointestinal water expressed in terms of predicted T.B.W. at post mortem in 13 human subjects are presented in Table V. "Total" gastrointestinal water comprised 1.4 per cent of the predicted T.B.W. The stomach, small intestine and the proximal half of the large intestine were found to contain an average of 0.4, 0.7 and 0.3 per cent of predicted T.B.W., respectively. "Total" intraluminal water content varied only from 0.5 to 2.2 per cent of T.B.W. In contrast, the intraluminal pool of the rabbit comprised 12 per cent of T.B.W.

DISCUSSION

The purpose of these experiments was to study the magnitude and exchange characteristics of intraluminal gastrointestinal water.

Total body water and the volume of intraluminal water in healthy animals might be expected to vary with body weight, age, sex and duration of fasting and thirsting. The animals studied were all young adults, each weighing about 2 Kg. Total body water was approximately 75 per cent of body weight in both male and female rabbits. The absence of a difference in T.B.W. between sexes contrasts with the significantly higher total body water content in males noted in studies on man (10). It is likely that these findings are explained by the fact that the female rabbits were all young nulliparous adults. Prepubertal human females have been shown not to differ from male subjects in body water content (10, 12). Cizek (3), in studies of somewhat larger and older rabbits, did find a significantly higher total body water in male than in female rabbits.

The volume of intraluminal water was not affected by the sex of the animal nor by fasting and thirsting up to 4 hours. The magnitude of this transcellular pool was approximately 12 per cent of T.B.W., which corresponds well with previous measurements (3) and represents a large fraction of the body water content of this species. This volume is comparable to one-half the volume of interstitial fluid or to twice the volume of plasma (13). The size of this subdivision of body water raises the possibility that it may contribute significantly to changes in plasma-interstitial fluid volumes induced by physiological or pathological influences. Furthermore, calculations of the distribution of water and ions which are based on a more simplified concept of the anatomy of body water, *i.e.*, a two-compartment system, will be erroneous in proportion to the volume of transcellular fluid in the species under study.

The observation that the S.A.R. of gut water to plasma water reaches unity in all segments within 4 hours indicates that this transcellular pool of water is in exchange equilibrium with the remainder of T.B.W. D_2O exchange is fastest in large bowel contents, while slower exchange occurs in small bowel contents, and the slowest exchange occurs in stomach water. If this isotope penetrated stomach mucosa only and then passed down the intestinal tract, water in the large bowel should equilibrate last. Our data effectively exclude this possibility and suggest instead that water penetrates across the mucosa of the gut throughout the length of the tract. The delay of

TABLE V

Intraluminal gastrointestinal water content in the human studied post mortem

Sex	Pathological diagnosis	Post-mortem interval (*hours*)	Age (*yrs.*)	Body weight (*Kg.*)	Predicted T.B.W. (*liters*)	Stomach			Small intestine			Cecum and proximal transverse colon			"Total" G.I.		
						(*ml.*)	(*ml./Kg.*)	(% *T.B.W.*)	(*ml.*)	(*ml./Kg.*)	(% *T.B.W.*)	(*ml.*)	(*ml./Kg.*)	(% *T.B.W.*)	(*ml.*)	(*ml./Kg.*)	(% *T.B.W*)
F-1	Recent myocardial infarction	6	77	35.0	22.4	51	1.5	0.2	100	2.9	0.5	13	0.4	0.1	164	4.8	0.8
M-2	Rheumatic heart disease with mitral stenosis	11	53	51.8	34.4	65	1.3	0.2	75	1.5	0.2	44	0.9	0.1	184	3.7	0.5
M-3	Hypertensive cardiovascular disease	22	51	43.6	29.7	233	5.4	0.8	335	7.7	1.1	33	0.8	0.1	601	13.9	2.0
F-4	Pituitary tumor	9	54	58.2	26.8	32	0.6	0.1	202	3.5	0.8	60	1.0	0.2	294	5.1	1.1
M-5	Squamous cell carcinoma of lung	7	64	43.6	29.7	233	5.4	0.8	151	3.5	0.8	13	0.3	0.1	397	9.2	1.7
F-6	Cerebral arteriosclerosis with encephalomalacia	6	56	58.2	26.8	132	2.3	0.5	60	1.0	0.2	178	3.1	0.7	370	6.4	1.4
F-7	Hypertensive and arterio-sclerotic heart disease	22	68	63.4	28.0	11	0.2	0.1	234	3.7	0.8	80	1.3	0.3	325	5.2	1.2
M-8	Paralysis agitans and bronchopneumonia	16	69	63.2	37.7	64	1.0	0.2	349	5.5	0.9	430	6.8	1.1	843	13.3	2.2
F-9	Cerebral thrombosis	20	67	65.0	29.9	226	3.5	0.8	95	1.5	0.3	—	—	—	321	5.0	1.1
M-10	Dissecting aneurysm of ascending aorta	19	59	68.0	38.0	34	0.5	0.1	241	3.5	0.6	20	0.3	0.1	295	4.3	0.8
M-11	Recurrent myocardial infarction	16	72	64.5	38.0	97	1.5	0.3	352	5.5	0.9	15	0.2	0.1	464	7.2	1.3
M-12	Chromophobe adenoma of pituitary	7	50	70.0	37.3	170	2.4	0.5	220	3.1	0.6	107	1.5	0.3	497	7.0	1.4
M-13	Traumatic demyelinization of cervical spinal cord	12	76	54.5	38.6	181	3.5	0.5	345	6.3	0.9	7	0.1	0.1	533	9.9	1.5
					Mean:	118	2.2	0.4	206	3.8	0.7	83	1.4	0.3	407	7.4	1.4
					Range:	11–233	0.2–5.4	0.1–1.2	60–352	1.0–7.7	0.2–1.1	7–430	0.1–6.8	0.1–1.1	164–843	3.6–13.8	0.5–2.2

TABLE VI

Summary of intraluminal gastrointestinal sodium, potassium, chloride, and water content in the rabbit

	Sodium		Potassium		Chloride		Water		Calculated concentration in intraluminal water*		
	M ±s.d. (*mEq.*)	M ±s.d. (% Na_e)	M ±s.d. (*mEq.*)	M ±s.d. (% K_e)	M ±s.d. (*mEq.*)	M ±s.d. (% Cl_e)	M ±s.d. (*ml.*)	M ±s.d. (% *T.B.W.*)	Sodium (*mEq./L.*)	Potassium (*mEq./L.*)	Chloride (*mEq./L.*)
Stomach	0.8 ±0.4	0.9 ±0.4	0.7 ±0.3	0.7 ±0.3	8.7 ±3.4	11.7 ±4.4	65 ±15	4.1 ±0.9	12	11	134
Small intestine	3.1 ±1.1	3.2 ±1.2	1.2 ±0.4	1.2 ±0.4	1.8 ±0.4	2.5 ±0.7	31 ±12	2.0 ±1.0	100	39	58
Cecum and transverse colon	10.0 ±2.0	10.2 ±2.1	4.5 ±3.1	4.5 ±2.7	1.2 ±0.3	1.7 ±0.4	93 ±22	6.0 ±1.6	108	48	13
"Total" G.I. content	13.7 ±2.4	14.2 ±2.4	7.1 ±2.8	7.2 ±2.2	11.8 ±3.6	16.0 ±4.5	189 ±40	12.1 ±2.7			

* Calculated concentrations are derived by dividing intraluminal sodium, potassium, and chloride contents by intraluminal water content. Each quantity was determined in separate series of animals.

equilibration in stomach water, where 4 hours was required for distribution equilibrium compared with 1 and 2 hours for colon and small bowel, respectively, may be a result of at least three factors. The ratio of membrane surface area to intraluminal volume may be smaller in stomach than in either large or small bowel. The ratio of surface to volume has been proposed as the basis for D_2O exchange rates in other transcellular pools (14). The delay of equilibration of labelled water in small bowel contents compared to large bowel contents cannot, however, be explained on comparative ratios of surface area to volume. A second possible explanation is that mucosal blood flow, and consequently the rate of delivery of isotope in proportion to the volume of intraluminal water, may be highest in the large bowel and least in the stomach (15). Finally, active transport of water across gut mucosa may occur and account for some of these differences (16).

In the course of these studies it was noted that the contents of the cecum and the proximal transverse colon were semiliquid and that in the mid-transverse colon there was a sharp transition zone, 1 to 2 cm. in length, where the contents were transformed into hard, dry pellets of stool. This would suggest that the mucosa of the mid-transverse colon acts to conserve water efficiently.

Data from previous experiments in which the intraluminal content of sodium, potassium, and chloride were determined are summarized in Table VI (4, 5, 7). The last three columns in Table VI show the calculated concentrations for each of these ions in intraluminal water of stomach, small bowel and large bowel. It is apparent from these values that the concentration of sodium, potassium and chloride maintained in intraluminal water bears no direct relation to the electrolyte structure of extracellular fluid. It would seem that their concentration and abundance in intraluminal water are determined by autonomous mechanisms in the gastrointestinal tract.

The presence of 14 per cent of Na_e, 7 per cent of K_e and 16 per cent of Cl_e in the contents of the gastrointestinal tract in rabbits has important implications in body partition studies where the normal anatomy of ion distribution or of ion shifts is measured (4, 5, 7). Calculating the extracellular fluid volume from a chloride space and assuming that all chloride exists in the same concentration as in plasma will lead to significant errors. Changes in the extracellular space inferred from changes in chloride concentration in plasma and external chloride balance may also be misleading since these calculations are based on the assumption that all, or nearly all, of the body chloride is in the plasma-interstitial fluid volume.

The volume of intraluminal water found in the gastrointestinal tract of man was a much smaller fraction of T.B.W. than in the rabbit. The significance of this species difference cannot be determined from our data since the observations on

human subjects must be evaluated cautiously for several reasons. Total body water was predicted from data on normal subjects; in contrast, accurate measurements were made in the rabbits. Significant migration of water from the gut may occur in critically ill patients. Post-mortem changes in intraluminal volume may have taken place during the 6 to 22 hours that elapsed between death and autopsy in these subjects. The measurements made in the human subjects consequently are not reliable, and further studies are needed to establish the amounts of intraluminal water and electrolytes in normal man.

SUMMARY

The volume of intraluminal gastrointestinal water was measured in rabbits and in human subjects studied post mortem. In rabbits this volume was referred to T.B.W. as determined by D_2O dilution. In man the intraluminal gut water was referred to predicted T.B.W. values.

Total body water averaged 75 per cent of the body weight in rabbits; 12 per cent of T.B.W. was contained in the lumen of the "total" gastrointestinal tract, with 4 per cent in the stomach, 2 per cent in the small intestine and 6 per cent in the large intestine. No significant difference between sexes was noted in either total body water or the volume of intraluminal gut water. Deuterium oxide equilibration was complete in large bowel water and nearly complete in small bowel water in 2 hours, but required 4 hours for completion in stomach water. The significance of delayed D_2O equilibration in stomach water compared with more distal segments of bowel was discussed with respect to the sites and mechanisms of D_2O exchange across gastrointestinal membranes.

The gastrointestinal tract of man at post-mortem examination contained approximately 1.5 per cent of the predicted T.B.W. The mean values were 0.4 per cent for stomach, 0.7 per cent for small bowel and 0.3 per cent for proximal large bowel. These values cannot be considered to represent the volume of intraluminal gut water to be found in the normal living human subject.

The amounts of intraluminal gut sodium, potassium, chloride and water in the rabbit are summarized in tabular form.

ACKNOWLEDGMENT

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THE CHEMICAL ESTIMATION OF ACYL GLUCURONIDES AND ITS APPLICATION TO STUDIES ON THE METABOLISM OF BENZOATE AND SALICYLATE IN MAN

By DAVID SCHACHTER[1]

(From the Department of Pharmacology-Biochemistry, School of Aviation Medicine, USAF, Randolph Air Force Base, Tex.)

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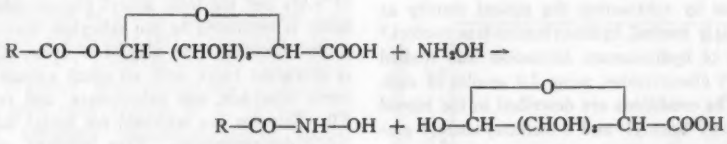
Structurally, glucuronides are the condensation products of hydroxyl-bearing compounds with the first, hemiacetal carbon of D-glucuronic acid. Two groups have been described (1). Alcohols and phenols form *ethereal* glucuronides, which are resistant to hydrolysis by mild alkali and do not reduce alkaline copper reagents. Carboxylic acids form *ester* glucuronides, which are easily split by mild alkali to liberate free glucuronic acid, a reducing agent. The term *acyl glucuronide* is introduced here for members of the latter group to denote clearly conjugation via the carboxyl group.

Another chemical reaction characteristic of the acyl glucuronides is described in this report. At room temperature and neutral pH, the acyl group can be transferred to hydroxylamine to form characteristic hydroxamic acids (Reaction I).



On addition of acid ferric chloride solution, hydroxamic acids yield colored products which can be measured spectrophotometrically (2). Thus, conversion to the stable hydroxamates provides a sensitive, chemical method for estimating the relatively unstable acyl glucuronides. The derivatives are easily extracted into organic solvents, and can be identified by chromatographic and other techniques. Non-acyl glucuronides do not form hydroxamic acids.

The methods reported here have permitted more extensive studies on the metabolic fate of aromatic acids in man. Since urinary glucuronides have been found following the ingestion of benzoate, salicylate, and probenecid (1, 3), observations were made on the metabolism of these clinically important drugs.

METHODS AND MATERIALS

Drug experiments. The subject was a healthy 28-year-old white male, weighing 60 kilos. His diet was not rigidly controlled, but remained fairly uniform throughout the period of investigation; all experiments were begun in the fasting state. At least 2 weeks elapsed between successive doses of drug. On the morning of an experiment, a control urine was collected over a period of several hours, and the appropriate drug was ingested as a solution of its sodium salt. Subsequently, timed voided urine samples were tested immediately for acyl glucuronides and stored at 5° C until completion of the remaining estimations. When necessary, the excretory rates of urinary drug metabolites were corrected for pre-ingestion, endogenous rates.

Estimation of acyl glucuronides. These compounds are estimated by conversion to their hydroxamates, using modifications of the procedure of Lipmann and Tuttle (2). The pH of the neutralized hydroxylamine reagent is critical and should be 7.0 ± 0.2. Stock solutions of NaOH (14 per cent) and hydroxylamine hydrochloride (28 per cent) are titrated against each other with a Beckman glass electrode pH meter to determine the exact proportions required. Urine samples containing 0.5 to 2.0 μmoles of acyl glucuronide are incubated with 0.5 ml. of freshly prepared neutral hydroxylamine for 2 hours at room temperature before color development and comparison with authentic hydroxamate standards. Figure 1 demonstrates the complete conversion of benzoyl glucuronide to benzoyl hydroxamate under these conditions.

[1] Present address: Department of Medicine, College of Physicians and Surgeons, New York City, N. Y.

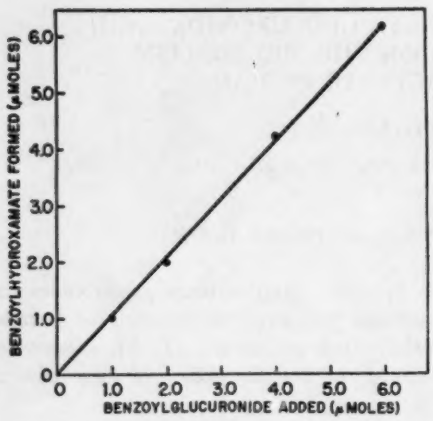


FIG. 1. QUANTITATIVE CONVERSION OF BENZOYL GLUCURONIDE TO BENZOYL HYDROXAMATE

The indicated amounts of crystalline benzoyl glucuronide in 0.5 ml. water were mixed with 0.5 ml. neutral hydroxylamine and incubated 2 hours at room temperature. The volume was adjusted to 2.0 ml. with water, and 0.5 ml. of 3 N HCl followed by 0.5 ml. of 5 per cent $FeCl_3 \cdot 6H_2O$ in 0.1 N HCl added. The optical density at 540 mμ was compared with that of a benzoyl hydroxamate standard.

To estimate salicyl acyl glucuronide (SAG) in urine by this procedure, 6 N HCl is substituted for 3 N HCl to minimize the formation of purple products arising from salicylate and salicylurate. Further, a correction is made in each estimation by subtracting the optical density at 540 mμ of a similarly treated, hydroxylamine-free control.[2]

The specificity of hydroxamate formation was studied with a series of 9 glucuronides, using 2.0 μmoles of each in the assay. (The conditions are described in the legend to Figure 1). Only benzoyl- and o-methoxybenzoyl glucuronides yielded such products, and no reaction was observed with the glucuronides of salicylamide, N-acetyl salicylohydrazine, 3-hydroxycoumarin, pregnandiol, phenolphthalein, menthol, and borneol.

Identification of urinary acyl glucuronides. Urinary hydroxamate-forming materials appear after the administration of benzoate and salicylate. The following evidence identifies these as benzoyl- and salicyl acyl glucuronides (SAG), respectively. These compounds are completely hydrolyzed by 0.1 N NaOH in 10 minutes at room temperature, and 84 per cent hydrolyzed by incubation with bacterial β-glucuronidase (250 units per ml.) for 60 minutes at 38° C. A single hydroxamate spot was detected for each compound by paper chromatography of urinary aliquots, using Whatman No. 1 paper and the ascending technique, with water-saturated n-butanol : glacial acetic acid (80 : 20, v/v) as the solvent system. These spots reduce aniline phthalate (4), and give a positive carbazole reaction for hexuronic acid (5) after elution into water. The benzoate metabolite moves at a rate (Rf 0.67) similar to that of crystalline benzoyl glucuronide. The salicylate conjugate (Rf 0.72) liberates free salicylate on hydrolysis in 6 N HCl at 100° C for 1 hour.

The hydroxamate derivatives of these urinary metabolites were extracted at neutral pH into ether and chromatographed on paper as described above, with water-saturated n-butanol as the developing solvent. Movement of the benzoyl derivative (Rf 0.79) corresponded to that of an authentic sample of benzoyl hydroxamate, and that of the salicyl compound (Rf 0.85) to crystalline salicyl hydroxamate.

That the urinary SAG is a monoglucuronide has been established in the following manner: its hydroxamate is completely extractable into ether, while that of the diglucuronide would be ether-insoluble. In addition, the hydroxamate is chromatographically homogeneous and identical in behavior with synthetic salicyl hydroxamate.

Estimation of urinary salicyl metabolites. In addition to SAG, salicyl phenolic glucuronide (SPG), salicylurate, and total salicyl were estimated.

SPG accounts for the difference between the quantity of SAG and the total salicyl glucuronide excreted. The latter is estimated by the salicylate liberated by bacterial β-glucuronidase. An aliquot of urine, adjusted to pH 2, is extracted twice with an equal volume of ether to remove salicylate and salicylurate, and readjusted to pH 6.0. Samples are withheld for initial salicylate (6) and SAG determinations. Five hundred units of β-glucuronidase are added to each ml. of extracted urine, the mixture is incubated at 38° C for 8 hours, an identical amount of enzyme again added and the incubation repeated.[3] Finally, 10 per cent perchloric acid filtrates are prepared and the free salicylate concentrations again determined.

$$\text{SPG} = \text{final salicylate} - \text{initial salicylate} - \text{SAG}$$

Salicylurate is estimated by a paper chromatographic technique. Suitable volumes of urine are quantitatively applied in duplicate to Whatman No. 1 paper for ascend-

[2] A satisfactory method of eliminating blank values due to salicylate and salicylurate is to extract the reaction mixture after formation of salicyl hydroxamate with 4 volumes of ether at neutral pH. Salicylate and salicylurate remain behind, while over 90 per cent of the hydroxamate is extracted. An aliquot of the ether is evaporated and color developed with HCl and ferric chloride. Values for acyl glucuronide in salicyl urine were identical, when estimated by both this procedure and that described in the text.

[3] Enzyme-treated urine was further hydrolyzed in 6 N HCl at 100° C for 3 hours, and free salicylate again estimated. The results indicated that enzymatic hydrolysis was at least 80 per cent complete under the conditions described in the text. Enzymatic hydrolysis is preferable to acid hydrolysis since specificity for glucuronides is obtained.

ing chromatography, with n-butanol: ethanol (40: 11, v/v) saturated with an ammonium carbonate buffer (7) as the solvent system. The dried paper chromatograms are viewed with an ultra-violet lamp, and the fluorescent salicylurate areas (Rf 0.31) outlined and eluted into water. (The salicylate fluorescent area corresponds to Rf 0.66.) To 2.8 ml. of eluate, containing about 0.5 μmole salicylurate, 0.2 ml. of 0.1 M ferric chloride in 0.07 N HCl is added. The optical density of the purple products is measured at 540 mμ in the Beckman model B spectrophotometer. Recoveries of known quantities of salicylurate, chromatographed simultaneously, have ranged from 90.0 to 102.0 per cent.

The procedure of Lester, Lolli, and Greenberg (6) was followed in estimating total urinary salicyl.[4]

Corrected total salicyl = observed total salicyl + salicylurate × 0.676 × 0.225 + salicylurate × 0.676 × 0.775 − salicylurate × 0.676 × 0.775 × 0.84

Corrected total salicyl = observed total salicyl + salicylurate × 0.236. The salicylurate concentration is estimated by the chromatographic method described in the text.

Other methods. Chemical estimations were employed for hippurate (9), hexuronic acid (5), and probenecid (3). Probenecid conjugates were hydrolyzed by refluxing in 4.0 N H_2SO_4 for 60 minutes. Optical density measurements were made in the Beckman model B spectrophotometer.

Materials. The following compounds were prepared according to published methods and recrystallized from hot water: benzoyl glucuronide (10), m.p. 181–2° (decomp.) (Pryde and Williams: 183° [11]); salicyluric acid (12), m.p. 166° (Quick: 167° [12]); salicyl hydroxamic acid (13), m.p. 168° (Jeanrenaud: 168° [13]). A reference standard solution of benzoyl hydroxamate was prepared by reaction of neutral hydroxylamine with recrystallized benzoic anhydride (14). Crystalline salicylamide glucuronide (174°), o-methoxybenzoyl glucuronide (123–4°), and N-acetyl salicylohydrazine glucuronide (208–10°) were gifts from Doctor R. T. Williams to Captain R. M. Dowben; 3-hydroxycoumarin glucuronide (207–8°) was a gift from Captain R. M. Dowben; menthol-, borneol-, pregnandiol-, and phenolphthalein glucuronides were purchased from the Sigma Chemical Company, as was the bacterial β-glucuronidase.

[4] In this procedure aliquots of salicyl urine are hydrolyzed to liberate salicylic acid, which is extracted into ether and estimated by reaction with ferric chloride. The "total salicyl" concentration so measured requires complete hydrolysis of all salicyl conjugates. However, 67.6 per cent of the glycine conjugate remains unhydrolyzed, as determined by the liberation of glycine (8) in similarly treated salicyluric acid solutions. Hence it is necessary to correct the observed total salicyl concentration for: 1) incomplete ether extraction of salicylurate (77.5 per cent as compared to 100 per cent for salicylate), and 2) relative intensity of the colored products formed with ferric chloride (84.0 per cent for salicylurate as compared to salicylate).

RESULTS

Ingestion of benzoate

In individual experiments, the subject was fed 6.9, 13.9, 34.7, and 69.3 millimoles of sodium benzoate.[5] Complete elimination of the drug as urinary hippurate and benzoyl glucuronide ensued in each instance, with final recoveries of 98 to 104 per cent. The relative amounts of benzoate conjugated with glycine and with glucuronic acid varied with the dosage, as shown in Figure 2. The glucuronide moiety increased progressively from 0.4 per cent (0.03 millimoles) to 3.0 per cent (2.08 millimoles). Hippurate accounted for the bulk of conjugated benzoate throughout this dose range.

The cumulative urinary recovery of each metabolite is plotted in Figure 3 for a representative experiment. The recoveries of acyl glucuronide and hippurate ran a parallel course, a characteristic observed at all the dosage levels examined. Complete excretion of benzoate required 3 to 4 hours after the two smaller doses, and 10 to 14 hours after the larger amounts.

As indicated in Table I, the maximal urinary excretory rate (μmoles per minute) achieved in a given experiment by either metabolite depended on the dose of benzoate. Limiting values for hip-

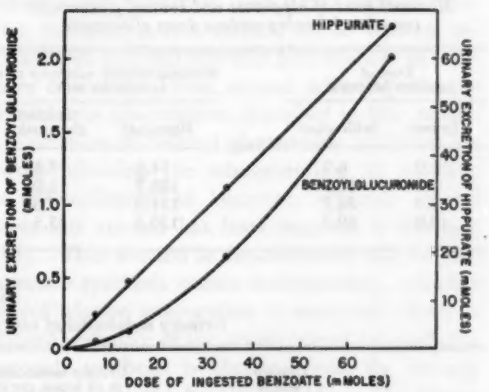


FIG. 2. QUANTITIES OF URINARY HIPPURATE AND BENZOYL GLUCURONIDE RECOVERED AFTER VARIOUS DOSES OF BENZOATE

The right- and left-hand vertical scales refer to hippurate and benzoyl glucuronide, respectively.

[5] Corresponding to 1.0, 2.0, 5.0, and 10.0 grams.

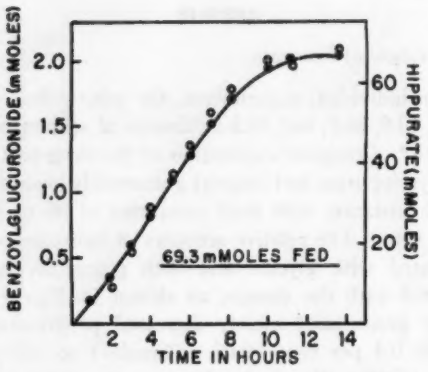


FIG. 3. CUMULATIVE URINARY RECOVERIES OF HIPPURATE (—●—●—) AND BENZOYL GLUCURONIDE (○)

purate excretion were approached at a dose of 13.9 millimoles. Quick (15) has demonstrated that limitations in the availability of glycine may account for this, and the peak rate attained in these experiments (130.5 μmoles per minute) is within his range for maximal rates of glycine mobilization in man (122 to 155 μmoles per minute). By contrast, the maximal excretory rate of benzoyl glucuronide was approximately proportional to the benzoate dose throughout the range studied. It is significant that the glucuronide was detected at a dosage level (6.9 millimoles) far below that required for maximal hippurate excretion. These observations fail to support the supposition (15) that the glucuronide synthesis is a reserve detoxication mechanism, operating only when the glycine supply is exceeded. Further pertinent evidence is obtained from the data in Figure 3. After the seventh hour in this experiment, the excretion of hippurate was well below its maximal rate, yet significant benzoyl glucuronide excretion persisted.

TABLE I

Maximal rates of hippurate and benzoyl glucuronide excretion following various doses of benzoate

Dose of sodium benzoate		Maximal urinary excretory rate (*μmoles per min.*)	
(*grams*)	(*millimoles*)	Hippurate	Benzoyl glucuronide
1.0	6.9	54.8	0.4
2.0	13.9	108.2	1.0
5.0	34.7	111.3	2.6
10.0	69.3	130.5	5.5

Ingestion of salicylate

Four experiments were performed, the subject ingesting 7.5, 15.0, 22.5, and 30.0 millimoles of sodium salicylate.[6] The relative quantities of urinary salicyl metabolites excreted after these doses are listed in Table II. With doses of 15.0 to 30.0 millimoles about 50 per cent of the amount fed was recovered as urinary salicyl in 24 hours (and 85 per cent at the end of 48 hours). Salicylurate made up 60 per cent of this, and the glucuronides 30 per cent, values which agree closely with previous observations (12, 16). SAG and SPG were present in about equal amounts. Cumulative urinary recoveries of the salicyl metabolites, in a representative experiment, are depicted in Figure 4. Whereas total salicyl, salicylurate, and SAG ran a course parallel to each other with time, the recovery of SPG was relatively lower in the early hours and higher thereafter. The maximal rates of excretion (μmoles per minute) of the urinary products varied with the doses, as shown in Table II; limiting values for these rates were not achieved in this range.

Characteristic differences in the excretory patterns of SAG and SPG were observed in all the

[6] Corresponding to 1.0, 2.1, 3.1, and 4.2 grams.

TABLE II

Urinary metabolites of salicylate following various oral doses

Dose (*millimoles*)	Urinary salicyl recovered in 24 hours, *per cent of dose*	Urinary metabolites recovered in 24 hours, per cent of total urinary salicyl			Maximal rates of urinary excretion (*μmoles per min.*)			
		Salicyl-urate	SAG	SPG	Total salicyl	Salicyl-urate	SAG	SPG
7.5	78.6	64.5	13.1	12.7	4.5	3.0	0.8	0.6
15.0	54.5	62.8	14.8	21.0	5.6	4.1	1.0	1.4
22.5	49.4	74.0	14.3	15.1	8.0	6.0	1.5	1.5
30.0	50.8	59.6	16.4	12.1	11.1	6.8	1.9	1.7

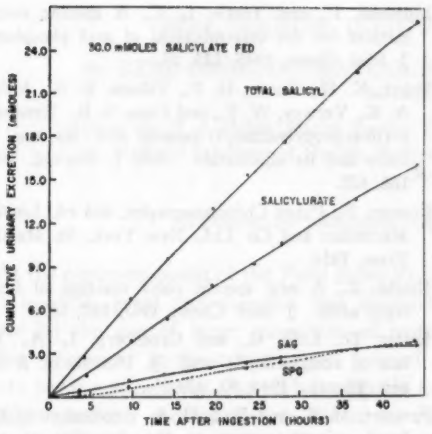


FIG. 4. CUMULATIVE URINARY RECOVERIES OF SALICYL METABOLITES

experiments. These are illustrated in Figure 5. The peak SAG excretory rates were observed within 5 to 10 hours after ingestion, with a rapid fall thereafter. By contrast, the SPG peak was obtained after 20 to 30 hours, and the subsequent fall-off was more gradual.

Ingestion of probenecid

Seven and one-half millimoles (2.1 grams) of this drug (sodium p-(dipropylsulfamyl) benzoate) were administered to the subject. At the end of

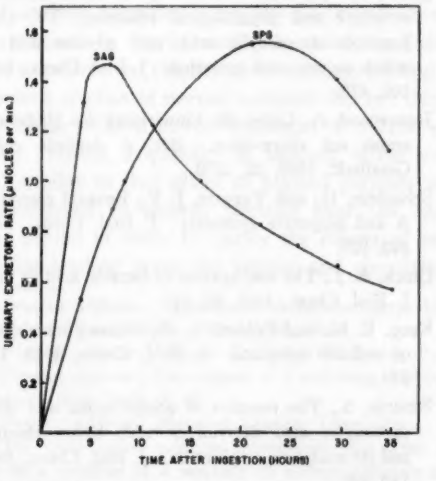


FIG. 5. RATES OF URINARY EXCRETION OF SAG AND SPG AFTER INGESTION OF SALICYLATE

the first, second, third, and fourth days, the cumulative urinary recoveries were 35.1, 59.1, 73.8, and 79.1 per cent. Consistently, 80 per cent appeared as the acyl glucuronide. The excretion of total hexuronic acid, estimated by the carbazole method, was equivalent to that of acyl glucuronide. By contrast, the urinary hexuronic acid concentration following benzoate was over three times that of benzoyl glucuronide, and after salicylate twice as much as that of the combined glucuronides. It thus seems likely that probenecid glucuronide is much less susceptible to hydrolysis in the body than the glucuronides of benzoate and salicylate.

DISCUSSION

Previous investigators have described the transfer to hydroxylamine of acyl groups linked to acids as anhydrides (2), or to alcohols as esters (17). It is of interest, therefore, that the acyl glucuronides, which constitute an intermediate category with acyl groups linked to a hemiacetal-bearing carbon, also form hydroxamates. This provides a sensitive method for their chemical estimation, and further characterizes them as a unique group within the class of glucuronides.

Conversion to the stable hydroxamates offers the advantage of a trapping mechanism for the readily hydrolyzed acyl glucuronides. These hydroxamates are readily extracted, purified, and subsequently identified. As a differential reaction, the hydroxamate method allows separate estimation of acyl and non-acyl glucuronides in mixtures of both. These several advantages made possible the observations described in this report.

Excretion of benzoyl glucuronide could be detected following the administration of as little as 6.9 millimoles of benzoate, although several times this amount has been required in the past (15). Thus it could be demonstrated that the glucuronide synthesis occurs independently, whether or not glycine conjugation is maximal. Only a minimal estimate of the actual rate of benzoyl glucuronide synthesis is obtained from the urinary recovery, as noted by Quick (15). The extent to which this conjugate may be broken down in the body is suggested by the three-fold excess of urinary hexuronic acid relative to benzoyl glucuronide. Therefore, it seems inappropriate to designate benzoyl glucuronide formation as merely a spare detoxication mechanism.

Following the oral administration of salicylate in this subject, three metabolites—salicylurate, SAG, and SPG—accounted for 90 per cent or more of the total urinary salicyl recovered. Other authors (6, 16) have reported the excretion of 20 to 25 per cent unconjugated salicylate under roughly comparable conditions, a difference possibly due to the breakdown of labile SAG, particularly in alkaline urines collected over prolonged periods. Kapp and Coburn (16) noted the urinary excretion of both acid- and alkaline-labile salicyl glucuronides,[7] and estimated their quantities. These authors suggested a diglucuronide structure for these conjugates, a possibility which the present results do not support. Of special interest was their subject W. J., who excreted equal amounts of the two glucuronides in the first 12 hours after intravenous salicylate, but almost twice as much acid-labile as alkaline-labile material in the succeeding 12 hours. These results clearly reflect the characteristic differences in the urinary excretory patterns of SAG and SPG described in the present studies.

SUMMARY

1. Acyl glucuronides react with hydroxylamine to form hydroxamic acids. This reaction may be applied to their estimation, purification, and identification.

2. Benzoate, salicylate, and probenecid elicited the urinary excretion of their respective acyl glucuronides when ingested orally by a normal subject.

3. Doses of sodium benzoate were quantitatively recovered as urinary hippurate and benzoyl glucuronide.

4. At least 90 per cent of various doses of salicylate was recovered as urinary salicylurate, salicyl acyl glucuronide, and salicyl phenolic glucuronide. Characteristic differences in the urinary excretory patterns of salicyl acyl glucuronide and salicyl phenolic glucuronide were described.

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[7] SAG is alkaline-labile, and SPG acid-labile.

ORAL SODIUM LOADING IN NORMAL INDIVIDUALS

By KEHL MARKLEY,[1] MANUEL BOCANEGRA,[2] GUILLERMO MORALES,[3] AND MIGUEL CHIAPPORI[4]

(From the U. S. Public Health Service, U. S. Department of Health, Education, and Welfare, National Institutes of Health, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.)

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At the commencement of the Peru Burn Project (1) in 1951, great apprehension was aroused in many by the use of large quantities of isotonic saline solutions[5] in the treatment of burned patients, for it was believed that such therapy in individuals retaining sodium due to the stress reaction would overload the circulation and lead to death in pulmonary edema. The reason for this fear was based upon the knowledge that man, in contrast to the dog, retained a great part of the sodium administered as isotonic sodium chloride over a considerable period of time (2, 3). Yet, during the course of the study of 110 burned children and 83 burned adults to whom had been given orally a quantity of an isotonic saline solution equivalent to 10 per cent of body weight during the first 24 hours after thermal injury and half that amount during the second 24-hour period, no untoward effects due to sodium, such as vomiting, generalized edema or massive pulmonary edema, were encountered (1), in spite of the fact that these patients demonstrated marked water and sodium retention (largely the result of accumulations in the burned area as edema fluid). The experimental conditions of sodium loading in previous studies of normal subjects did not duplicate those utilized in the treatment of burned patients Hence, a quantity of isotonic saline solution similar to that given to burned patients was administered to normal persons over the same time period in order to clarify the effects of such sodium loading upon the kidney and circulation.

[1] Present address: Department of Biochemistry, University of Pennsylvania, Philadelphia, Pa.

[2] Present address: Manuel Candamo 475, Lima, Peru.

[3] Present address: Department of Cardiology, Bellevue Hospital, New York, N. Y.

[4] Present address: Hospital Loayza, Lima, Peru.

[5] The term 'saline solution' throughout this paper refers to a solution of a mixture of sodium chloride and sodium bicarbonate. For exact composition, see Materials and Methods.

MATERIALS AND METHODS

Renal function. Twelve healthy Peruvian males between the ages of 20 and 28 years were studied. None had a medical history of previous renal or cardiovascular disease. The subject was not permitted to take any liquid or food after 8 P.M. of the day previous to the performance of the study. After voiding on arrival at the hospital at 8 A.M., he was weighed nude and placed in bed in a recumbent position. The bladder was catheterized, venous pressure was measured carefully in the antecubital vein, and three twenty-minute clearance periods using inulin and sodium para-aminohippurate (PAH) by the technique of Goldring and Chasis (4) were accomplished. Five hundred cc. of water had been given approximately one and one-half hours previous to the measurement of the first clearance period, and suprapubic pressure and injection of air were used to empty the bladder. The catheter was then removed. At 11 A.M., after the subject had voided, a gastric tube was introduced into the stomach via the nose, and a constant drip of an isotonic saline solution containing 137 mEq. per L. of Na, 93 mEq. per L. of Cl and 44 mEq. per L. of HCO_3 was administered by the gastric tube at a uniform rate of 4 to 5.5 cc. per min. Each subject was given a total quantity of saline solution equivalent to 10 per cent of body weight over a period of 21 hours, during which time the subject remained recumbent and received no food or water. At 2-hour intervals the subject voided spontaneously, and the urine volume was measured and the urine saved. At 8 A.M. the next morning, when the total quantity of saline solution had been given, he was again weighed after voiding and then catheterized. Venous pressure was measured, and three clearance periods of 15 to 20 minutes each were repeated with inulin and PAH, this time without giving water previously. At the termination of the clearance studies the subject was weighed once more.

In the urine and plasma collected during the clearance periods PAH was analyzed by the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber (5), inulin by the method of Schreiner (6), sodium and potassium by the flame photometer using lithium as internal standard (7), and chloride by the method of Van Slyke and Hiller (8). In the first blood sample drawn at the control period and the period after completion of the sodium loading test, hematocrit was determined by the Wintrobe method, total plasma protein by the copper sulfate method (9), alkaline reserve by the manometric

method of Van Slyke (10), and concentration of sodium and potassium in red blood cells separated from plasma after one-half hour centrifuging at 3,000 rpm by the flame photometer method. Sodium and potassium concentrations were determined also in the 2-hour urine samples collected. Afterwards, the separate 2-hour samples of urine were mixed and the total quantity of urine excreted over the period of sodium administration was analyzed for sodium and potassium. Glomerular filtration and renal plasma flow were calculated during the control period and at the termination of the oral sodium loading. Total quantity of sodium and potassium excreted (UV) during the same periods, as well as during the 2-hour collection periods, was calculated. In addition, percentage of filtered sodium excreted was calculated during all clearance periods.

Plasma volume study. Eleven healthy Peruvian males between the ages of 20 and 28 years (including seven of the former subjects who volunteered for this test one month after completion of the previous one) were studied. All of the same conditions were maintained as before, except for the fact that no renal clearances were performed. Instead, at 9 A.M. on the day of the beginning of the test, plasma volume was measured by the four sample techniques using T-1824 (11). Again the subjects received a quantity of the saline solution equivalent to 10 per cent of body weight via gastric tube uniformly by constant drip over a 21-hour period. After completion of administration of the saline solution (8 A.M.), plasma volume determination was repeated as on the day before.

RESULTS

Hemodynamics

In Table I are listed glomerular filtration rate, renal plasma flow and plasma volume during the control period and during the period just after the administration of a quantity of the isotonic saline solution equivalent to 10 per cent of body weight. In eleven of the twelve subjects the glomerular filtration rate increased significantly over the control value and in only one individual did it stay the same. The average value shows a significant increase (+ 32 per cent) over the control value after the sodium load. On the other hand, renal plasma flow increased over the control value in six of the twelve subjects, decreased in five and stayed the same in one. The average value of all the subjects does not show a significant increase over the control value. The plasma volume increased over the control value in four of twelve individuals, decreased in six and remained the same in one after the administration of the saline solution. The average value of all the subjects shows no significant change over the control value. Venous pressure was measured during the control period (average, 104 mm.) and after sodium load-

TABLE I

Hemodynamic studies in normal subjects with oral sodium loading

Subject	No. of clearances	Glomerular filtration *cc./min./1.73 m²*			Renal plasma flow *cc./min./1.73 m²*			Plasma volume *cc.*		
		Control*	After*	Diff.	Control*	After*	Diff.	Control	After	Diff.
J. C.	3	105	161	55	614	725	111	3,055	3,358	303
M. C.	2	160	253	93	599	925	326			
L. M.	2	129	195	66	752	730	−22	2,110	2,400	290
P. L.	2	162	198	36	1,099	1,375	276			
E. R.	3	130	126	−4	648	577	−71	2,610	2,483	−127
A. R.	3	108	139	31	759	587	−172	3,354	3,600	246
A. V.	3	138	167	29	668	668	0			
J. G.	3	103	146	43	635	605	−30			
V. F.	3	118	163	45	631	750	119			
L. Q.	3	145	186	41	825	863	38	3,027	2,731	−296
R. C.	3	102	133	31	685	649	−36	3,035	3,048	13
C. G.	3	114	137	23	728	768	40	2,800	2,650	−150
A. L.								3,510	3,097	−413
J. R.								2,256	2,320	64
J. E.								2,720	2,455	−265
J. M.								2,941	2,463	−478
Average values		126	167	41†	720	768	48	2,856	2,782	− 74
Standard error of average				6.9			40.7			84

* Each number represents the average value of the clearance periods.
† Significant at the 1 per cent level.

TABLE II

Water and electrolyte excretion in normal subjects with oral sodium loading

Subject	No. of clearance periods	Urine volume* cc./min.		$U_{Na}V$* mEq./min.			U_{Na}* mEq./L.		U_KV* mEq./min.		U_K* mEq./L.	
		Control	After	Control	After	Sum	Control	After	Control	After	Control	After
J. C.	3	2.65	4.22	0.345	0.660	1.005	135.3	158.8	0.0689	0.0390	26.4	9.3
L. M.	2	3.79	3.03	0.310	0.520	0.830	85.7	172.0	0.0425	0.0449	11.7	14.8
P. L.	2	4.88	4.40	0.560	0.934	1.494	118.8	212.5	0.0913	0.0892	19.8	20.3
M. C.	2	9.75	3.85	0.526	0.718	1.244	54.1	187.0	0.0911	0.0294	9.4	7.6
E. R.	3	3.80	6.53	0.485	0.701	1.186	130.6	113.2	0.1168	0.0684	31.2	10.8
A. R.	3	4.02	3.65	0.528	0.572	1.100	148.4	155.5	0.0473	0.0277	13.1	7.6
A. V.	3	3.96	4.15	0.403	0.794	1.197	103.0	192.9	0.1021	0.0543	26.2	13.2
J. G.	3	4.69	1.63	0.286	0.396	0.682	64.4	242.0	0.0642	0.0437	14.1	26.7
V. F.	3	4.14	4.43	0.457	0.758	1.215	125.6	176.3	0.0586	0.0701	16.4	16.5
L. Q.	3	1.66	4.01	0.422	0.751	1.173	265.0	91.6	0.0367	0.0260	23.0	4.9
R. C.	3	1.36	5.04	0.345	0.841	1.186	49.4	167.1	0.0390	0.0400	4.9	8.0
C. G.	3	5.29	9.33	0.613	0.961	1.574	124.7	103.2	—	—	—	—
Average value		4.16	4.52	0.440	0.717		117.1	164.3	0.0690	0.0484	17.8	12.7

Analysis of variance of $U_{Na}V$ †

Source	df	Sum of squares	Mean square	F
Mean	1	8.0342		
Total	23	0.8804		
Control vs After	1	0.4611	0.4611	68.82 ‡
Subjects	11	0.3455		
Interaction	11	0.0738	0.0067	
				$F_{.01}$ 9.65

* Each number represents the average value of the clearance periods.
† $U_{Na}V$ = Total Na excretion; U_{Na} = Na concentration in urine excreted.
U_KV = Total K excretion; U_K = K concentration in urine excreted.
df = Degrees of freedom.
‡ Significant at the 1 per cent level.

ing (average, 133 mm.) in nine individuals, but no significant change was noted.

Excretion of water and electrolytes

In Table II are shown the concentrations and total quantity of the ions, Na and K, excreted during the clearance periods performed before and after the administration of the saline solution. The concentrations of sodium increased significantly after sodium loading, while the concentration of potassium decreased. The total quantity of sodium excreted augmented significantly after sodium loading (+ 63 per cent), while potassium diminished (− 30 per cent). The sodium excreted during the control period represented 2.58 per cent of the filtered load, while the sodium excreted after the administration of the sodium load represented 3.33 per cent of the filtered load. Although the volume of urine increased in seven of twelve individuals, the average urine volume after sodium loading showed no significant increase over the

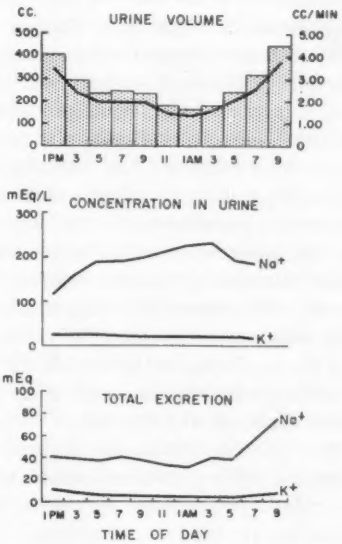

FIG. 1. DIURNAL VARIATIONS WITH ORAL SODIUM LOADING

TABLE III

Fluid and electrolyte balance in normal subjects with oral sodium loading *

Subject	Fluid intake cc.	Electrolyte intake mEq. Na	K	Cl	Urinary output cc.	Urinary excretion mEq. Na	K	Weight change Kg. After Na load	3 hrs. after Na load
M. C.	6,650	822	0	558	2,620	432	40	+2.1	+1.6
L. M.	5,300	685	0	465	3,340	455	43	+1.5	+0.7
	5,300	726	0	493	4,000	480	46	+0.5	
P. L.	6,000	822	0	558	4,860	690	53	+0.3	−1.1
E. R.	5,000	685	0	465	3,020	382	38	+1.7	+0.9
	5,400	740	0	502	2,540	315	62	+1.7	
A. R.	6,000	810	0	534	2,925	375	22	+2.4	+1.4
	6,000	810	0	534	960	232	38	+4.4	
A. V.	6,600	904	0	614	4,960	660	66	0.0	−1.1
J. G.	7,000	959	0	651	2,000	340	68	+3.3	+2.9
V. F.	6,000	822	0	558	3,600	490	59	+0.7	−0.5
L. Q.	6,000	822	0	558	3,500	517	39	+1.5	
	6,000	822	0	558	2,680	493	66	+2.2	
R. C.	7,000	959	0	651	3,300	609	64	+3.3	+2.5
	7,000	959	0	651	3,060	536	59	+3.3	+2.1
C. G.	6,300	863	0	586	3,900	554	78	+1.1	−0.4
	6,000	822	0	558	2,780	378	98	+2.0	
J. R.	5,000	685	0	465	1,540	408	55	+1.8	
J. E.	5,300	726	0	493	4,540	654	91	+0.3	
J. M.	6,300	863	0	586	1,540	442	31	+2.9	
Average	6,000	815	0	552	3,083	472	56	+1.85	+0.82

* Table does not include one subject in which the sodium load produced diarrhea and another who had large saliva loss.

control value due to the fact that the control value itself represented a slight water diuresis.

In Figure 1 are demonstrated the average urine volume, concentration of sodium and potassium and total excretion of sodium and potassium of the 2-hour urine collections during the administration of the saline solution. The first 2-hour sample showed an increase of water and sodium excretion as compared to the values prior to the administration of the saline solution. Water, sodium and potassium excretion all decreased during the night hours with sodium excretion rising notably during the early morning hours above the values encountered at the beginning of the test on the previous day. Sodium concentration in the urine rose during the administration of the sodium load with only a moderate effect noticeable due to changes in water excretion, while potassium concentrations remained relatively constant regardless of water excretion.

In Table III are listed the total intake and total output of water and electrolytes during the administration of the sodium load, together with the changes of weight affected by the sodium administration. It is noted that 51 per cent of the water and 58 per cent of the sodium administered were excreted via the kidney during the period of oral administration of sodium. From the weight data, it can be observed that at the completion of the sodium loading only 31 per cent of the water administered (1.85 Kg.) was retained, which rapidly diminished to 14 per cent (0.82 Kg.) three hours after the termination of the sodium administration. The different responses of a single subject receiving the same sodium load on two separate occasions are also given.

Blood analyses

Table IV demonstrates the changes in hematocrit, total plasma proteins, plasma concentration of sodium, potassium, chloride and bicarbonate and erythrocyte concentration of sodium and potassium before and immediately after the administration of the sodium load. Although some of

TABLE IV

Blood analyses in normal subjects with oral sodium loading

Analyses	Control Average ± S.D.	After Na loading Average ± S.D.
Hematocrit, %	46.2 ± 2.48	44.0 ± 7.2
Tot. Proteins, *gm.* %	7.66 ± 0.34	7.17 ± 0.47*
Plasma Na, *mEq./L.*	139.1 ± 3.51	137.2 ± 3.39
Plasma K, *mEq./L.*	4.13 ± 0.37	3.86 ± 0.30*
Plasma Cl, *mEq./L.*	101.3 ± 0.95	100.7 ± 0.55
Plasma HCO_3, *mEq./L.*	24.6 ± 0.41	23.0 ± 0.51*
RBC K, *mEq./L.*	90.8 ± 3.5	87.9 ± 4.2*
RBC Na, *mEq./L.*	14.5 ± 0.35	14.7 ± 1.84

* The difference between the control value and the value after the sodium load has statistical significance.

these values changed significantly due to the administration of the large quantities of saline solution, none of the changes appear to be of any real importance.

DISCUSSION

The excretion of 58 per cent of a large oral sodium load during the period of its administration challenges the widely held belief that man, unlike the dog, is sluggish in excreting sodium (2). Since previous studies in the literature concerned with sodium loading in man have all been performed giving smaller total amounts of physiologic sodium chloride solution (1 to 3 liters), but by more rapid intravenous infusion (13 to 65 cc. per min.), it is possible that the previously unreported experimental conditions used in this study are responsible for the observed difference.

Earlier investigators have held that man excreted sodium sluggishly compared to the dog due to the fact that glomerular filtration did not increase in man with the intravenous administration of sodium chloride. In the present study, however, there was usually a consistent and significant increase of glomerular filtration at the termination of oral sodium loading. Since the percentage of filtered sodium excreted increased significantly from 2.58 per cent to 3.33 per cent, it is evident that decreased tubular reabsorption over and above the increased amount filtered by the glomerulus accounted for a part of the augmented sodium excretion. Even though it is difficult to compare the results of this study with prior ones, it can be stated, nevertheless, that water and sodium excretion after oral sodium loading fell to values between those reported in non-prehydrated (2) and prehydrated (12) subjects maintained in the recumbent position.

The magnitude of the sodium and water diuresis after oral sodium loading is masked somewhat by the control values, since these latter values themselves demonstrated a slight water and sodium diuresis compared to data in the literature. The slight water diuresis during the control period was due to the administration of a small quantity of water before the commencement of the test. The slight sodium diuresis is more difficult to explain, but is most likely due to an augmented ingestion of sodium in the daily Peruvian diet prior to the test.

Although no attempt was made to study renal hemodynamics during the course of the administration of the sodium load, the gross data of sodium and water excretion on the 2-hour samples collected during that time demonstrated the well-known diurnal variations of water and sodium excretion without sodium loading, but at higher levels than reported under normal circumstances (13).

SUMMARY

1. Sixteen healthy young Peruvian males were given a quantity of an isotopic saline solution equivalent to 10 per cent of body weight in the recumbent position by constant drip via a gastric tube over a period of 21 hours. Renal hemodynamics and electrolyte excretion were studied in twelve of the subjects and plasma volumes in eleven of them before and after the administration of the sodium load.

2. Glomerular filtration rate increased an average of 32 per cent over the control value after administration of the sodium load, while effective renal plasma flow did not change significantly.

3. While body weight was increased significantly at the termination of the sodium load, plasma volume did not change significantly. The increase in body weight largely disappeared within 3 hours after termination of the load.

4. During the period of administration of the sodium load, 58 per cent of the administered sodium and 51 per cent of the administered water were excreted via the kidneys. The concentration and total quantity of sodium excreted in the urine increased significantly at the termination of the sodium load. An increased filtration of sodium by the glomeruli as well as a decreased reabsorp-

tion of sodium by the tubules are considered to be responsible for this phenomenon. Water excretion was augmented also, but its significance was masked by the slight water diuresis present in the control values. The diurnal variations of sodium and water excretion during the course of administration of the sodium load were evident, but at higher levels than under normal circumstances.

5. Total excretion of potassium as well as potassium concentration in the urine decreased after sodium loading.

6. There were no important changes in plasma concentrations of red blood cells, total proteins, or electrolytes.

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OBJECTIVE EVALUATION OF PATIENTS WITH RHEUMATIC DISEASES. II. PAPER ELECTROPHORETIC STUDIES OF SERUM GLYCOPROTEIN AND PROTEIN FROM PATIENTS WITH RHEUMATOID ARTHRITIS[1]

By GEORGE STIDWORTHY, R. W. PAYNE, CLARA L. SHETLAR, AND M. R. SHETLAR

(From the Research Laboratory, VA Hospital, and the Departments of Biochemistry and Pharmacology, University of Oklahoma School of Medicine, Oklahoma City, Okla.)

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Serum glycoprotein concentrations serve as a valuable index of the intensity of inflammatory activity in the rheumatic diseases (1), particularly when the glycoprotein level is considered in relation to the serum protein with which it is bound, *i.e.*, as the polysaccharide protein ratio (PR). The typical changes of serum protein (lowering of albumin, increase of globulin) in rheumatoid arthritis are well known (2).

The development of techniques for serum protein and glycoprotein analyses by filter paper electrophoresis permits a detailed study of the changes in each of the serum protein components as well as the distribution of carbohydrate bound to these components (3). The relationship of these various components to the severity of inflammatory activity of rheumatoid arthritis has consequently been investigated.

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METHODS

Laboratory. Paper strip electrophoresis was performed essentially as described by Block, Durrum, and Zweig (4) using Durrum type cells (Spinco Model R). Electrophoretic runs were made at room temperature (78° to 85°) for 16 hours on Whatman 3MM filter paper strips saturated with 0.075 M Barbital buffer (pH 8.6) using a constant current of 5 milliamps for each cell of 8 strips. A volume of 10 microliters was applied to the paper for protein fractionations and 30 microliters for glycoprotein separations. All samples were run in duplicate. Protein was visualized on the paper strips by staining with bromophenol blue-zinc sulfate as described by Block, Durrum, and Zweig (4). The glycoprotein color development on the paper strips was accomplished by the Periodic-acid Schiff reaction largely as described by Koiw and Gronwall (5). However, the reducing solution was prepared as described by Roboz, Hess, and Forster (6).

Quantitation of the developed strips was made with a servo type recording photometer and automatic integrator (Spinco Model R Analytrol). Protein strips were scanned through blue filters, glycoprotein strips through green filters (Klett 52). The total area under the protein curve was equated to the total serum protein as determined by the biuret reaction of Weichselbaum and Shapiro (7), and the glycoprotein curve to the total serum glycoprotein as determined by the tryptophan

TABLE I

Serum protein fractions as estimated by paper electrophoresis

Group	No.	Protein as per cent of total protein of				
		Albumin	α_1	α_2	β	γ
Normal	13	57.3 ±1.7‡	4.9 ±0.3	9.4 ±0.7	11.9 ±0.6	16.3 ±1.3
Rheumatoid arthritis						
Clinical activity 1	8	49.5*±0.8	5.3 ±0.4	10.6 ±0.6	13.7 ±0.1	20.9 ±1.4
Clinical activity 2	13	44.9*±1.2	6.4*±0.4	11.1*±0.4	14.3*±0.6	23.3*±1.2
Clinical activity 3 and 4†	12	39.5*±2.0	6.8*±0.3	14.7*±0.5	15.4*±0.6	23.7*±2.3

* Significantly different from the normal group at the 1% level of probability.
† Composed of eight with activity 3 and four with activity 4.
‡ Figures following the ± sign are standard errors of the mean of each group.

TABLE II

Protein bound polysaccharide† of serum fractions as estimated by paper electrophoresis

	No.	Mg. % of polysaccharide of						
		Total	Albumin	α_1	α_2	β	γ	Seromucoid
Normal	13	115 ±4.1	14.8±0.7	16.3 ±0.9	33.6 ±2.3	28.3 ±1.3	22.5±1.3	11.0 ±1.4
Rh. arthritis								
Clin. activity 1	8	150*±3.6	14.7±1.1	19.7*±1.3	51.9*±2.5	35.9*±1.6	27.8±1.8	20.5*±1.6
Clin. activity 2	13	176*±14.6	14.3±1.8	28.0*±1.7	66.6*±2.9	36.7*±1.4	29.0±2.5	30.5*±1.7
Clin. activity 3 and 4‡	12	197*±5.9	14.2±0.9	34.5*±3.0	81.1*±3.9	37.6*±3.2	29.1±2.2	30.3*±0.8

	No.	Polysaccharide as per cent of the protein (PR) of					
		Total protein	Albumin	α_1	α_2	β	γ
Normal	13	1.48 ±0.03	0.33 ±0.02	4.27 ±0.28	4.67 ±0.30	3.04±0.21	1.81±0.09
Rh. arthritis							
Clin. activity 1	8	1.91*±0.03	0.37 ±0.05	4.84*±0.11	6.29*±0.35	3.47±0.31	1.79±0.21
Clin. activity 2	13	2.26*±0.06	0.41 ±0.05	5.89*±0.16	7.87*±0.44	3.42±0.16	1.61±0.13
Clin. activity 3 and 4‡	12	2.73*±0.06	0.52*±0.04	6.96*±0.40	7.93*±0.32	3.32±0.26	1.77±0.13

* Significantly different from the normal group at the 1 per cent level of probability.

† Bound hexose as determined by the tryptophan method (8). Hexosamine component of the polysaccharide complex is not included.

‡ Composed of eight with activity 3 and four with activity 4.

method of Shetlar, Foster, and Everett (8). The quantitations of individual fractions were made by calculating the percentage of the total area contributed by the areas representing each of the various fractions. The areas representing each of the glycoprotein fractions were selected by comparing the glycoprotein densitometer curve with the corresponding protein curve.

Seromucoid was determined by the method of Weimer and Moshin (9).

Clinical. An estimate of clinical activity of the rheumatoid process was assigned at each clinic visit as described in a previous report (10). Current disease activity was thus grossly appraised as:

Activity 1—No inflammatory activity
Activity 2—Mild activity
Activity 3—Moderate activity
Activity 4—Severe activity

RESULTS

Results of the analyses of the serum protein and glycoprotein fractions from patients with rheumatoid arthritis of various degrees of severity are summarized in Tables I and II. Typical electrophoretic strips of a normal serum are depicted in Figure 1, and those of a patient with severe rheumatoid arthritis in Figure 2.

With increasing severity of the rheumatoid process an increase occurred in all the globulin fractions, being most marked in the α_2 globulin. A corresponding significant decrease in the serum albumin fractions occurred; indeed it would appear that the decreasing serum albumin is a slightly more sensitive index of activity than the serum globulin increase.

The most striking changes in serum glycoprotein of patients with rheumatoid arthritis occurred in those carbohydrate moieties associated with the α_1 and α_2 protein fractions. This increase is in excess of the protein increase in the α_1 and α_2 fractions, resulting in an increased percentage of bound carbohydrate in these fractions (Table II). On the other hand the increases in the β and γ fractions merely parallel the changes in protein contents of these respective fractions.

Correlation coefficients of protein, seromucoid and glycoprotein fractions with clinical activity of the rheumatoid arthritis patients are presented in Table III. Clinical activity was found to have a significant positive correlation with total PR, α_1 and α_2 glycoprotein, seromucoid and α_2 globulin protein, and a negative correlation with serum albumin. Seromucoid exhibited significant correlation with total glycoprotein (expressed either as mg. per cent or as PR) and with α_1 and α_2 glycoprotein. The total PR was correlated with the PR's of the α_1 and α_2 globulins. Total glycoprotein (in mg. per 100 ml.) exhibited a low but sig-

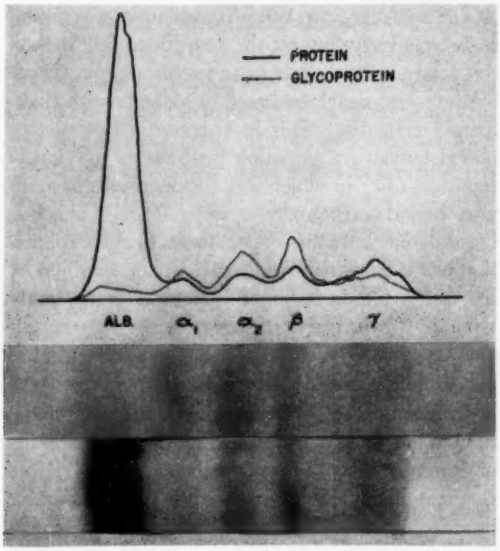

FIG. 1. TYPICAL PAPER STRIP ELECTROPHORETIC PATTERNS OF NORMAL SERUM WITH CORRESPONDING DENSITOMETER TRACINGS

The solid line indicates protein concentration; the dotted line, glycoprotein concentration. The top strip is stained with Periodic-acid Schiff reagents for glycoprotein and the bottom with bromophenol blue for protein.

The total hexose glycoprotein of this serum was 108 mg. per 100 ml. distributed among the fractions as follows: Albumin, 13.2 mg. per 100 ml.; α_1, 14.5; α_2, 27.6; β, 30.2; and γ, 22.4. The total protein of this sample was 7.42 grams per 100 ml.

nificant correlation with α_1 glycoprotein, but not with α_2 glycoprotein.

DISCUSSION

Changes in serum protein produced by various types of rheumatic diseases have been reported. The data of Table I are confirmatory of earlier work in which a decrease of serum albumin, and an elevation of the globulin fractions was noted. The observation that serum albumin as determined by a 26 per cent sodium sulfate salting out procedure has a negative correlation with activity of rheumatoid arthritis (11) is further confirmed by paper electrophoresis studies (Table III). The correlation coefficient of -0.526 for the salting out method is very close to the -0.563 found in the present study. Use of the electrophoretic method allows further investigation of relationships between clinical activity and protein fractions. The finding of a positive correlation of 0.635 for α_2 globulin with clinical activity is of interest, as elevations of this fraction have been noted in many inflammatory conditions. No other protein fraction was significantly correlated with activity. It may be implied that albumin and α_2 globulin fractions are more rapidly affected than are other fractions by inflammatory activity in rheumatoid arthritis insofar as their protein moieties are concerned.

The concentration of serum glycoprotein in patients with rheumatoid arthritis serves as an index of the inflammatory activity of this disease. As can be seen in Table II the most striking elevation of serum glycoprotein in patients with rheumatoid arthritis is found in carbohydrate bound

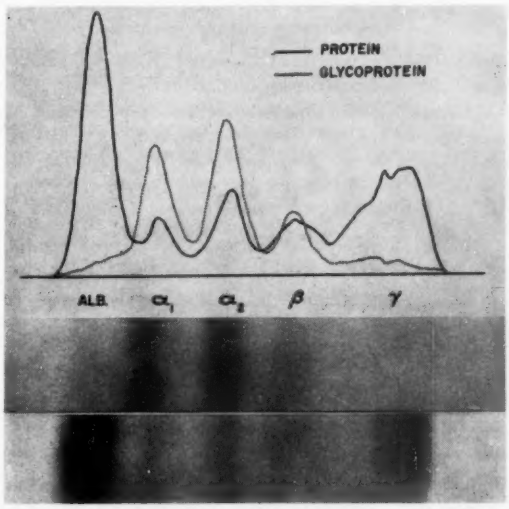

FIG. 2. TYPICAL PAPER STRIP ELECTROPHORETIC PATTERNS AND CORRESPONDING DENSITOMETER TRACINGS OF THE SERUM OF A PATIENT WITH ACTIVE RHEUMATOID ARTHRITIS

The solid line indicates protein concentration; the dotted line, glycoprotein concentration. The top strip is stained with Periodic-acid Schiff reagents for glycoprotein and the bottom strip with bromophenol blue for protein.

Total hexose glycoprotein of this serum was 208. mg. per 100 ml. distributed among the fractions as follows: Albumin, 9.6 mg. per 100 ml.; α_1, 63.4; α_2, 79.7; β, 33.7; and γ, 21.6. Total protein of this sample was 7.22 grams per 100 ml.

TABLE III

Summary of correlation coefficients

	r*	t Value†	P
Correlation with clinical activity			
Total PR‡	0.914	12.50	.01
α_1 PR‡	0.559	3.75	.01
α_2 PR‡	0.296	1.72	.10
Albumin % of total protein	−0.563	3.79	.01
α_1 % of total protein	0.110	0.64	.30
α_2 % of total protein	0.635	4.58	.01
β % of total protein	0.183	1.34	.15
γ % of total protein	0.306	1.79	.10
α_1 polysaccharide, mg. %	0.654	4.82	.01
α_2 polysaccharide, mg. %	0.684	5.21	.01
Seromucoid	0.689	3.49	.01
Correlation with seromucoid			
Total PR‡	0.786	5.09	.01
Total polysaccharide, mg. %	0.826	3.85	.01
Alb. polysaccharide, mg. %	−0.224	0.92	.25
α_1 polysaccharide, mg. %	0.826	5.88	.01
α_2 polysaccharide, mg. %	0.603	3.02	.01
β polysaccharide, mg. %	0.448	1.69	.15
γ polysaccharide, mg. %	−0.082	0.21	.45
Correlation with total polysaccharide			
α_1	0.459	2.88	.01
α_2	0.114	0.64	.35
Correlation with total polysaccharide protein ratio			
α_1 PR‡	0.686	5.24	.01
α_2 PR‡	0.706	5.55	.01

* r = Correlation coefficient.

† $t = \frac{n^{\frac{1}{2}}r}{(1 - r^2)^{\frac{1}{2}}}$ $n = N - 2$.

‡ Polysaccharide (bound hexose) as per cent of the protein involved.

to the α-globulins. This increase is in excess of α-globulin protein increases since there is a disproportionately large percentage of glycoproteins bound to both α_1 and α_2 globulins in the active phases of the disease studied. Some of this elevation is probably due to increases in the seromucoids which have electrophoretic mobilities at pH 8.6 similar to the α-globulins. Furthermore, the high correlation coefficients of α_1 globulin carbohydrate (0.826) and α_2 globulin carbohydrate (0.603) with seromucoid suggest a close relationship of these components in rheumatoid arthritis. However, the seromucoid levels (Table II) are not high enough to account for all of the carbohydrate increases found in the α_1 and α_2 fractions. One may well speculate that carbohydrate rich fractions with mobilities at pH 8.6 similar to α_1 or α_2 globulin, as yet not definitely characterized, are elevated in the sera of patients with rheumatoid arthritis.

The carbohydrate bound to albumin was found to be relatively low by the procedure followed in this study. Only slight changes of albumin carbohydrate percentage occurred in patients with rheumatoid arthritis. This is in contrast to data obtained by salt fractionation methods as previously reported (11) in which striking increases of albumin bound carbohydrate were found in active rheumatoid arthritis. The most likely explanation of the disparity between the data obtained by the two methods is that the albumin fraction obtained by salt fractionation contains appreciable amounts of carbohydrate rich globulin, and thus has a higher carbohydrate content.

The significant changes in serum α_2 globulin, the bound carbohydrate of α_1 and α_2 globulin and of seromucoid with increasing clinical activity of rheumatoid arthritis patients are of considerable interest. The technique of filter paper electrophoresis would appear to have advantages in that only minute amounts of serum are required for analysis and the entire spectrum of protein and glycoprotein may be investigated. However, data obtained from total serum polysaccharide-protein ratio as obtained by chemical methods, which are a necessary preliminary to paper strip quantitation, are apparently as satisfactory as any of the fractionation data for use in evaluating activity of the rheumatoid process.

SUMMARY

Paper electrophoretic techniques have been applied to analyses of serum protein and glycoprotein components from patients with rheumatoid arthritis of various degrees of severity. The increase of serum glycoprotein in patients with rheumatoid arthritis is due to 1) increases of the carbohydrate rich globulin fractions relative to the carbohydrate poor albumin fraction and 2) an increase of the carbohydrate content of the α_1 and α_2 globulin fractions.

Changes in the α-globulins are apparently most closely related to inflammatory activity in rheumatoid arthritis, as the carbohydrates associated with α_1 and α_2 globulin and the α_2 globulin protein increased with increasing clinical activity. A decrease of albumin protein occurred with increasing severity of the disease.

Total serum glycoprotein (expressed as hexose polysaccharide as a percentage of the serum protein) exhibited the highest correlation with clinical activity, and consequently is to be recommended as the most satisfactory laboratory method of those studied for evaluation of the status of patients with rheumatoid arthritis.

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THE ABSORPTION OF RADIOIRON LABELED FOODS AND IRON SALTS IN NORMAL AND IRON-DEFICIENT SUBJECTS AND IN IDIOPATHIC HEMOCHROMATOSIS

By ROBERT B. CHODOS,[1] JOSEPH F. ROSS,[2] LEONARD APT,[3] MYRON POLLYCOVE,[4] AND JAMES A. E. HALKETT

(From the Radioisotope Unit and Medical Service, Veterans Administration Hospital, Boston, the Department of Medicine, Boston University School of Medicine, the Department of Pediatrics, Harvard Medical School, and the Department of Medicine, Tufts University School of Medicine, Boston, Mass.)

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It is generally accepted that, in the absence of bleeding or pregnancy, approximately 1 mg. of iron is lost per day from the body (1–11). As a corollary, the quantity of iron in the body is largely determined by the amount of iron absorbed from the gastrointestinal tract. Fundamental knowledge of iron absorption has been obtained by balance studies (2, 4, 12–18), by determination of the increase in the serum iron level (19) or circulating hemoglobin (20–21) after oral iron administration, and by evaluation of the percentage of orally administered radioiron incorporated into hemoglobin (22–26) or accounted for in both hemoglobin and feces (7, 27).

The present investigation was undertaken to compare the absorption of egg and vegetable iron with that of iron salts fed to normal subjects, to patients with iron-deficiency, and to patients with idiopathic hemochromatosis. The method of study used was similar to that introduced by Dubach, Callender, and Moore (27). These investigators showed that the quantity of iron used in hemoglobin formation may not always be a true index of iron absorption (27, 28). Since a negligible quantity of iron is excreted into the intestinal tract (1–3, 7–8, 11) and since stool iron is almost entirely unabsorbed dietary iron (2), additional information concerning iron absorption may be obtained by determining both the percentage of orally administered radioiron incorporated into hemoglobin and the percentage recovered in the feces. The percentage of the oral dose not recovered in hemoglobin and feces may be considered an approximate measure of the quantity of iron deposited in tissue stores. In certain patients this figure may be subject to a significant experimental error due particularly to incomplete stool collection.

EXPERIMENTAL SUBJECTS AND METHODS

Three groups of patients were studied:

1. *Normal subjects.* This group consisted of 32 male patients with uncomplicated dermatitis, psychosomatic or psychiatric illnesses, or neurologic diseases. All had normal hematologic and serum iron values. Iron stores were not specifically measured by tissue biopsy (bone marrow or liver). Blood loss was excluded by history and stool examination. Systemic diseases that might influence iron absorption, utilization, or the level of iron stores were excluded by appropriate laboratory and clinical evaluation.

2. *Subjects with deficient iron stores.* This group comprised 15 patients with evidence of chronic blood loss. The red blood cells were hypochromic and microcytic, serum iron levels were less than 50 micrograms per 100 ml. and a definite history of blood loss was obtained in each case.

3. *Subjects with excess iron stores.* This group of 9 patients had idiopathic hemochromatosis. The diagnosis was established by clinical features, elevated serum iron level, and by liver or skin biopsy.

The number of absorption studies and the form in which the iron was administered to these subjects are summarized in Table I.

Red blood cell indices were determined for each subject using equipment certified by the Bureau of Standards. Serum iron was measured by the method of Kitzes, Elvehjem, and Schuette (29). The whole blood, red cell, and plasma volumes were estimated using a radiophosphorus tagged red cell method (30).

Following these studies, tracer doses of radioiron were given by mouth to the fasting subject either as ferrous or

[1] Present address: VA Hospital, Syracuse, New York.

[2] Present address: School of Medicine, University of California, Los Angeles, California.

[3] Present address: Wills Eye Hospital, Philadelphia, Pennsylvania.

[4] Present address: Donner Laboratory, University of California, Berkeley, California.

TABLE I

Summary of iron absorption studies

Group	No.	Iron-59 administered				
				As labeled food		
		As $FeCl_3$	As $FeCl_3$	Eggs	Vegetables	Total no. of studies
Normal	32	25	2	14	6	47
Iron deficient	15	14	2	3	2	21
Hemochromatosis	9	13	0	1	2	16
Total	56	52	4	18	10	84

ferric chloride or as a radioiron labeled food. Ferrous chloride was prepared by the reduction of ferric chloride with powdered ascorbic acid or, in 2 studies, with cysteine. The oral dose of Fe^{59} [5] used in the iron salt absorption studies ranged from 12 to 50 microcuries, and from 7 to 23 microcuries in the food iron absorption studies.

In all but 7 studies evaluating the absorption of ferrous chloride, ascorbic acid tablets (total of 0.5 to 1.0 gm.) were administered concomitantly with the radioactive iron salt. A variable quantity of non-radioactive carrier iron ranging from 4 or 5 mg. to 80 mg. was usually added to the tracer dose. The smaller carrier doses were used if the absorption of food iron had been previously studied in the subject, since this dose range approximated the quantity of iron administered in the labeled foods. Larger carrier doses were used in the remaining iron salt absorption studies.

The Fe^{59} labeled foods were chicken eggs and vegetables. The techniques developed for preparation of these labeled foods will be reported separately (31). The quantity of iron in a given oral dose of labeled food was determined by the method of Kitzes, Elvehjem, and Schuette (29) on an aliquot of the food substance after Kjeldahl digestion with concentrated nitric, sulfuric, and perchloric acid. Representative iron content of the various foods used is tabulated in Table II.

Radioiron labeled foods were administered after a night's fast. Two pieces of bread (containing approximately 1 mg. of iron) without butter, and black coffee (no iron) with sugar were eaten with the labeled food. Eggs were served scrambled and vegetables were boiled and served with the cooking liquid. Eight ounces of orange juice were given with scrambled eggs in a single study.

TABLE II

Iron content of labeled food products

Food product	No. of assays	Iron content (*mg./100 gm.*)		Iron administered (*mg.*)	
		Range	Average	Range	Average
Chicken eggs	16	2.8–4.7	3.8	3.9–10.0	6.0
Swiss chard	5	0.6–0.9	0.8	2.2– 4.4	2.9
Beet greens	5	1.1–2.5	1.8	2.0– 4.8	3.0

[5] The radioactive iron used in these studies was prepared in the atomic pile at Oak Ridge. The specific activity ranged from 455 to 4,237 mc. per gm. Fe.

After iron administration, stools were collected until less than 1 per cent of the oral tracer dose was recovered in a 24-hour collection. Samples of blood were obtained at 2 to 5-day intervals. In most instances, blood samples were obtained until a plateau of constant activity was reached.

The preparation of the collected materials for counting was relatively simple. Water was added to feces in the large collecting bottles, total weight determined, and the mixture homogenized by an Osterizer or an Equipoise shaker. After mixing, three aliquots by weight were transferred to screw cap bottles holding 25 ml. of material. Since the quantity of radioiron present in plasma after the first 24 hours was not significant, blood samples were processed for assay by pipetting 25 ml. of whole blood into similar bottles. Appropriate standards representing the administered oral dose of radioactive iron salt or food iron were similarly prepared. The standard for iron salts consisted of a portion of the solution taken per mouth by the subjects while that for labeled foods was made up from weighed aliquots of scrambled eggs or cooked vegetables. These were digested in concentrated nitric acid and diluted to a 25-ml. volume in vials like those used for counting the stool and blood samples.

TABLE III

Recovery of radioiron added to feces

Experiment	No. of stool samples ctd.	Net cps total stool (ave.)	Net cps std. (ave.)	% Recovery
1	8	640.63	664.4	96.42
2	8	644.74	664.0	97.09
3	9	668.96	662.6	100.95
4	9	627.90	652.3	96.25

Gamma radiation of the prepared samples was quantitatively measured by Geiger-Mueller counting tubes. Initially, a Sylvania GG306, all metal, bismuth cathode tube was used. This heavily shielded tube was mounted in a horizontal position in a plastic frame and the sample bottles were placed as close as possible beneath the tube, with the long axis of the sample bottle parallel to the tube. Counts were recorded on a Tracerlab Autoscaler. The counting efficiency of this arrangement for Fe^{59} was 0.4 per cent and 180 cps represented 1 microcurie. Statistical analysis of the whole sampling, positioning, and counting technique revealed a potential error that did not exceed ± 5 per cent.

Later another type of Geiger-Mueller tube was used, the Texas Well Counter.[6] This tube was approximately 8 to 10 times as sensitive as the GG306. All food absorption studies utilized the Texas Well Counter, counts being recorded by a Berkeley Decimal Scaler.

[6] Welch-Allyn Company, Skaneateles, New York.

The accuracy of the gamma counting methods used for the assay of Fe^{59} in fecal and blood samples was determined by *in vitro* recovery experiments. A known quantity of Fe^{59} in the form of $FeCl_3$ was added directly to 4 different fecal collections. This material was homogenized, processed as above, and the amount of Fe^{59} present determined by gamma counting techniques. Data obtained were compared with activity observed in a water solution of Fe^{59} counted under identical circumstances. These data are summarized in Table III and reveal that 96 to 101 per cent of the added Fe^{59} were accounted for in this study. The presence of solid material in feces did not significantly alter the counts recorded.

RESULTS

Absorption of iron by normal subjects (Figures 1, 2, 3, and 4)

Figures 1 and 2 reveal a significant difference between the absorption of ferrous chloride and food iron. Recovery results in Figure 1 show approximately 4 to 12 per cent of radioiron were absorbed and incorporated into hemoglobin when Fe^{59} was administered as ferrous chloride along with 27 to 41 mg. of carrier ferrous iron. Sixty-

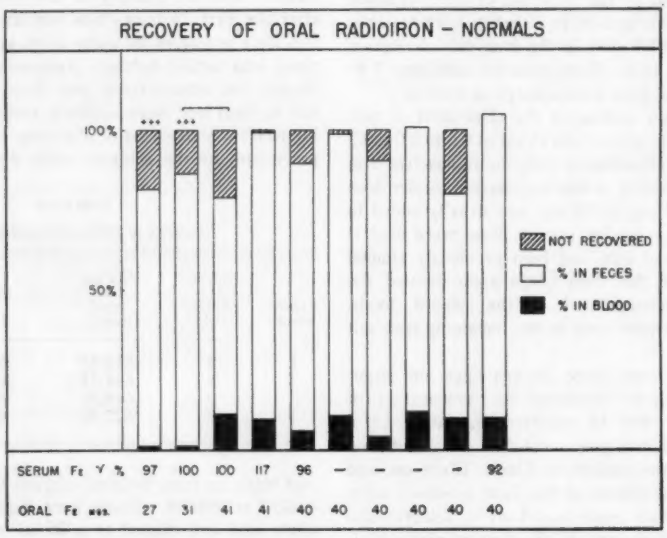
RECOVERY OF ORAL RADIOIRON – NORMALS
100%
50%
+++
+++
NOT RECOVERED
% IN FECES
% IN BLOOD
SERUM Fe γ % 97 100 100 117 96 – – – – 92
ORAL Fe mg. 27 31 41 41 40 40 40 40 40 40

FIG. 1. RECOVERY OF RADIOIRON BY NORMAL SUBJECTS FOLLOWING THE ORAL ADMINISTRATION OF AN IRON SALT

The labeled salt was administered as ferric chloride (+++ above column) or as ferrous chloride (no symbol above column) along with 27 to 41 mg. of non-radioactive carrier iron. The bracket ⟌ indicates multiple studies on a single patient.

It is to be noted that a portion of the oral dose was not recovered (cross hatched area). This unrecovered portion in this figure and in all subsequent figures represents a combination of three factors: absorbed iron not synthesized into hemoglobin but deposited in tissue stores, the error of determinations, and failure by the subject to make absolute quantitative stool collections.

Hematologic values for subjects

	Range	Mean
Hematocrit (%)	43.0–51.5	46.5
Hemoglobin (*gm.* %)	13.3–16.0	14.4
MCHC (%)	28.0–33.0	31.9

(MCHC—Mean corpuscular hemoglobin concentration)

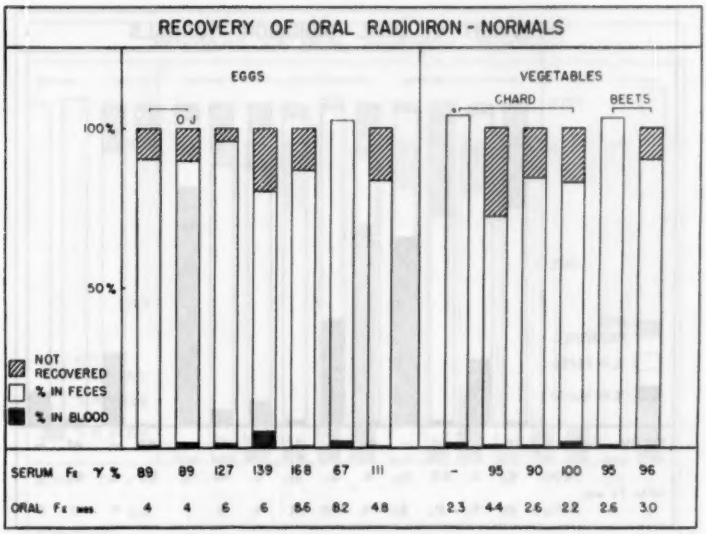

FIG. 2. RECOVERY OF RADIOIRON BY NORMAL SUBJECTS FOLLOWING THE ADMINISTRATION OF A RADIOIRON LABELED FOOD CONTAINING FROM 2.2 TO 8.6 MG. OF NON-RADIOACTIVE IRON

O. J. (Bar No. 2) indicates 8 ounces of fresh orange juice was administered with the radioiron labeled eggs.

Hematologic values for subjects

	Range	Mean
Hematocrit (%)	43.0–50.0	46.7
Hemoglobin (*gm.* %)	13.2–15.4	14.1
MCHC (%)	29.0–32.0	31.0

seven to 96 per cent were recovered in feces and 1 to 22 per cent were not recovered in blood or feces. The percentage of Fe^{59} incorporated into hemoglobin is indicative of the quantity of carrier iron definitely absorbed and used to form hemoglobin (0.9 to 4.6 mg.). As the legend to Figure 1 states, the unrecovered percentage of radioiron in this group and in all subsequent groups represents a combination of three factors: absorbed iron not synthesized into hemoglobin but deposited in tissue stores, the error of the determinations, and failure by the subject to make absolutely quantitative collection of stools.

The percentage of absorption and utilization of food iron (Figure 2) was significantly less than that of ferrous chloride even though the amounts of iron given were considerably smaller. With a single exception only 0.5 to 2.3 per cent of egg and vegetable radioiron were incorporated into hemoglobin. In one study 5 per cent of labeled egg iron was used to form hemoglobin. Sixty-five to 99.5 per cent were recovered in the feces and 4 to 28 per cent were not recovered in either the feces or blood. The addition of orange juice in one study did not increase the absorption of egg iron. The percentage of Fe^{59} absorbed and incorporated into hemoglobin indicated the absorption of from 0.03 to 0.3 mg. of the egg or vegetable iron administered. Calculations of the percentage of iron not recovered suggest that as much as 0.3 to 1.3 mg. of iron could have been added to tissue stores.

The greater absorption of the iron salt, ferrous chloride, is more apparent when compared with the absorption of similar quantities of ferrous chloride and egg iron (Figure 3). Seven normal subjects, who received Fe^{59} labeled eggs containing from 4 to 8 mg. of iron, absorbed and incorporated 0.5 to 5.0 per cent into hemoglobin, representing

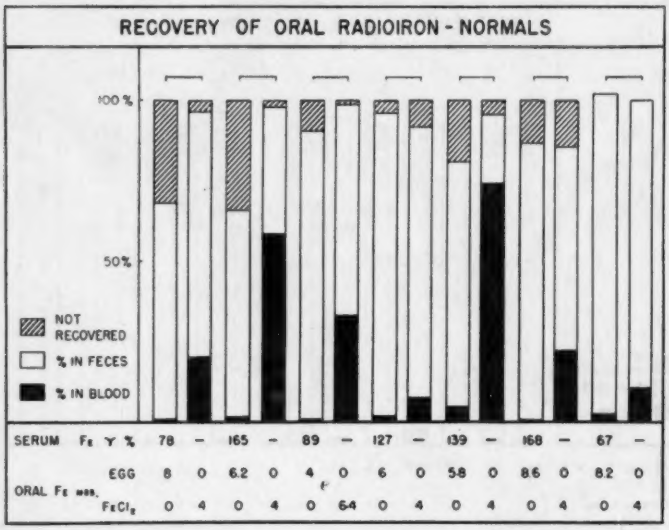
RECOVERY OF ORAL RADIOIRON - NORMALS
100 %
50%
NOT RECOVERED
% IN FECES
% IN BLOOD
SERUM Fe γ %
78 – 165 – 89 – 127 – 139 – 168 – 67 –
ORAL Fe mg.
EGG
8 0 6.2 0 4 0 6 0 58 0 8.6 0 8.2 0
FeCl3
0 4 0 4 0 64 0 4 0 4 0 4 0 4

FIG. 3. COMPARATIVE RECOVERY OF RADIOIRON BY NORMAL SUBJECTS FOLLOWING THE ADMINISTRATION OF 1) RADIOIRON LABELED EGGS, AND 2) A TRACER DOSE OF RADIOACTIVE FERROUS CHLORIDE

The carrier non-radioactive iron ranged from 4 to 8.6 mg.

Hematologic values of subjects

	Range	Mean
Hematocrit (%)	43.0–50.0	45.9
Hemoglobin (*gm.* %)	12.9–15.1	13.6
MCHC (%)	27.0–31.0	30.0

the absorption of from 0.08 to 0.3 mg. of iron. When a similar quantity of ferrous chloride was given, 7.8 to 74.5 per cent of the dose appeared in hemoglobin, representing the absorption of 0.32 to 3.0 mg. of iron. The increased absorption and incorporation of this iron salt into hemoglobin ranged from 2 to 100 times that of egg iron.

The effect of egg itself upon the absorption of iron was studied by giving inorganic Fe^{59}, as ferrous chloride, to five subjects along with non-labeled scrambled eggs (Figure 4). Under this experimental condition a considerably smaller percentage of iron was absorbed and utilized for hemoglobin formation than when a similar amount of ferrous chloride was administered alone. However, when compared to the absorption and utilization of egg iron, there was usually an appreciably greater absorption and use of the ferrous iron salt even in the presence of eggs. This difference ranged from 1.5 to 10 times the amount of food iron absorbed and incorporated into hemoglobin.

These data as summarized in Table IV indicate that iron in eggs and certain vegetables is not nearly as well absorbed as the iron salt ferrous chloride, and that the concomitant presence of food such as egg may decrease significantly the absorption of the iron salt.

Absorption of iron by subjects with deficient iron stores (Figures 5 and 6)

Representative results summarized in Figure 5 show that with one exception more than 20 per cent of the iron was absorbed and incorporated into hemoglobin when Fe^{59} was administered as ferrous or ferric chloride. Nearly all of the remaining radioiron was recovered in the stools except in a single patient with a complicating chronic infection (Figure 5, Studies 7 and 8).

The absorption of Fe^{59} labeled food by subjects with deficient iron stores is summarized in Figure 6. Food iron was not as well absorbed as iron

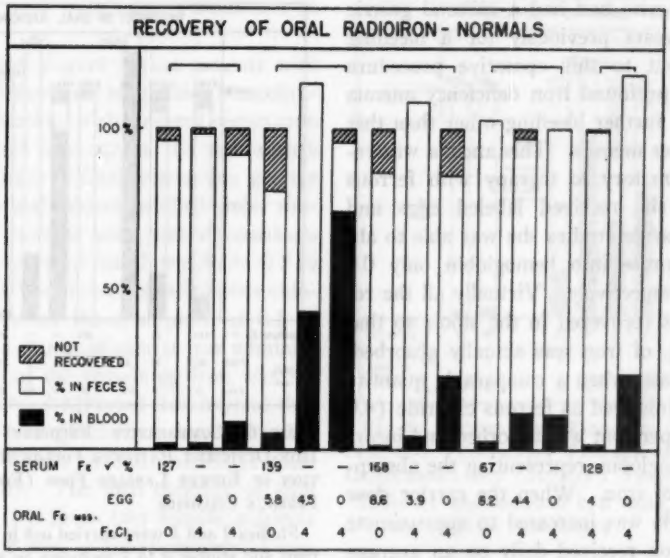
RECOVERY OF ORAL RADIOIRON - NORMALS
100%
50%
NOT RECOVERED
% IN FECES
% IN BLOOD
SERUM Fe ↓ %
127 — — 139 — — 168 — — 67 — — 128 —
ORAL Fe ass.
EGG
6 4 0 5.8 4.5 0 8.6 3.9 0 8.2 4.4 0 4 0
FeCl2
0 4 4 0 4 4 0 4 4 0 4 4 4 4

FIG. 4. THE COMPARATIVE RECOVERY OF RADIOIRON BY NORMAL SUBJECTS FOLLOWING THE ADMINISTRATION OF 1) A SINGLE FEEDING OF RADIOIRON LABELED EGGS, 2) A COMBINED FEEDING OF RADIOACTIVE FERROUS CHLORIDE AND NON-LABELED EGGS, AND 3) A TRACER DOSE OF RADIOACTIVE FERROUS CHLORIDE

The carrier non-radioactive iron ranged from 4 to 8.6 mgs.

Hematologic values of subjects

	Range	Mean
Hematocrit (%)	44.0–51.0	47.3
Hemoglobin (*gm.* %)	12.7–15.1	14.0
MCHC (%)	29.0–32.0	30.0

administered as ferrous chloride. Iron-deficient subjects receiving Fe^{59} labeled chicken eggs absorbed and utilized 10.3, 20.5, and 0.6 per cent of Fe^{59}, while those receiving Fe^{59} labeled vegetables absorbed and utilized 3.6 and 30 per cent. Most of the remaining Fe^{59} not appearing in hemoglobin was recovered in the stools.

TABLE IV

Per cent of radioiron incorporated into hemoglobin in normal subjects

Form of iron	No. of studies	Oral dose (*mg.*)	Per cent in hemoglobin		
			Range	Mean	Median
$FeCl_3$	2	27.0–31.0	0.8– 0.9	0.85	0.85
$FeCl_3$	12	20.0–41.0	3.9–11.6	8.73	9.80
$FeCl_3$	8	4.0– 6.4	7.8–74.5	31.40	23.25
Egg+$FeCl_3$	5	7.9– 8.4	1.3–43.2	12.60	3.90
Egg	14	4.0– 8.6	0.5– 5.0	1.40	0.60
Swiss chard	4	2.3– 4.4	0.8– 1.6	1.20	1.25
Beet greens	2	2.6– 3.0	0.8– 0.9	0.85	0.85

The quantity of carrier iron absorbed and incorporated into hemoglobin by patients with deficient iron stores ranged from 1.0 mg. when 2 to 10 mg. of food iron were given, to from 4.8 to 18.5 mg. when 31 to 80 mg. of iron were administered as ferrous or ferric chloride. These figures cannot be compared since similar quantities of iron were not administered. However, iron-deficient patients absorbed a much larger percentage of food iron than did normal subjects who received a similar quantity of food iron.

Multiple studies were carried out on two iron-deficient subjects to compare the absorption of ferrous chloride and labeled food. One patient (Figure 6, Studies 2 and 3) absorbed 20 per cent of Fe^{59} incorporated in eggs and 100 per cent of a comparable dose (5.0 mg.) of ferrous chloride. The second patient (Figure 6, Studies 4 through

7) was a female who had had a subtotal gastric resection three years previously for a bleeding ulcer. Subsequent to this operative procedure she developed a profound iron deficiency anemia in the absence of further bleeding other than that associated with her menses. This anemia was reported to be refractory to therapy with ferrous sulfate. When she received labeled eggs and chard in two separate studies she was able to absorb and incorporate into hemoglobin only 0.6 and 3 per cent, respectively. Virtually all the remaining Fe^{59} was recovered in the stools so that less than 0.1 mg. of iron was actually absorbed. In striking contrast, when a comparable quantity of Fe^{59} was administered as ferrous chloride (4.0 mg. of iron), 88 per cent was absorbed and incorporated into hemoglobin, representing the absorption of 3.5 mg. of iron. When the carrier dose of ferrous chloride was increased to approximate the 80 mg. of iron received daily on an average therapeutic regimen of ferrous sulfate, 32 per cent of the dose was absorbed and incorporated into hemoglobin, representing the absorption of 26 mg. of iron.

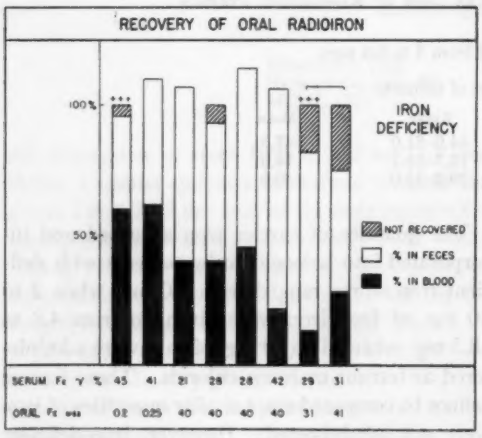
RECOVERY OF ORAL RADIOIRON
100%
50%
IRON DEFICIENCY
NOT RECOVERED
% IN FECES
% IN BLOOD
SERUM Fe γ % 45 41 21 28 28 42 29 31
ORAL Fe mg. 02 025 40 40 40 40 31 41

FIG. 5. RECOVERY OF RADIOIRON BY PATIENTS WITH DEFICIENT IRON STORES FOLLOWING THE ADMINISTRATION OF AN IRON SALT

The labeled salt was administered as ferric chloride (+++ above column) or as ferrous chloride (no symbol above column) with 31 to 41 mg. of carrier iron. No carrier iron was administered in studies 1 and 2.

Hematologic values of patients

	Range	Mean
Hematocrit (%)	21.1–44.0	20.8
Hemoglobin (*gm.* %)	5.0–13.0	8.4
MCHC (%)	22.4–30.0	27.0

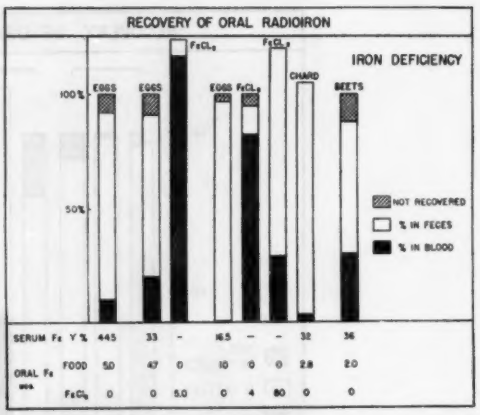

FIG. 6. COMPARATIVE RECOVERY OF RADIOIRON BY IRON-DEFICIENT PATIENTS FOLLOWING THE ADMINISTRATION OF EITHER LABELED FOOD (EGGS, VEGETABLES) OR FERROUS CHLORIDE

Studies 2 and 3 were carried out in sequence on one patient and studies 4 to 7 inclusive on a second patient.

Hematologic values of patients

	Range	Mean
Hematocrit (%)	29.0–40.0	32.0
Hemoglobin (*gm.* %)	6.0–11.9	7.5
MCHC (%)	19.0–30.0	23.0

In summary, iron as ferrous chloride is absorbed in larger quantity by iron-deficient subjects than is the iron in eggs and vegetables. However, food iron is absorbed and used to a much greater extent by patients with iron deficiency than by normal subjects.

Absorption of iron by subjects with excess iron stores (Figures 7 and 8).

Sixteen absorption studies have been performed on 9 patients with the excess iron stores of idiopathic hemochromatosis. Seven of these patients were males and two [7] were females past the menopause. The comparative absorption of ferrous chloride and Fe^{59} labeled eggs was evaluated in one patient with hemochromatosis. The absorption of iron before and after an intensive venesection program was measured in one patient with

[7] We wish to thank Drs. Alexander Marble and Frank Gardner, Boston, Massachusetts, for the opportunity to study these patients.

hemochromatosis, and only after venesection in another.

It is evident from Figure 7 that patients with well-established, untreated idiopathic hemochromatosis of long duration, with elevated serum iron levels and saturated iron stores did not absorb either ferrous chloride or food iron to any greater extent than did normal subjects studied under similar conditions. Patients with hemochromatosis absorbed and incorporated into hemoglobin 0.5 to 11.1 per cent of Fe^{59} administered as ferrous chloride. This represented the absorption of 0.1 to 3.3 mg. of the 24 to 41 mg. of carrier iron administered. Almost all of the remaining iron was recovered in the feces. Additional iron beyond that incorporated into hemoglobin may have been absorbed by the two female patients studied (Figure 7, Studies 7 and 8). The total quantity of iron possibly absorbed by these two female subjects was 28 and 29.7 per cent, or approximately 11 mg. of iron by each patient.

Absorption of food iron by untreated patients with hemochromatosis was evaluated in two patients who received Fe^{59} labeled beet greens containing 2.8 and 4.8 mg. of iron (Figure 7). Only 1.0 and 1.1 per cent of iron was absorbed and incorporated into hemoglobin. This represents absorption of a quantity of food iron which is essentially the same as that obtained in normal subjects. If all the unrecovered iron was absorbed and deposited in tissue, the total absorption could be increased to 22 and 18.1 per cent, or 0.6 and 0.9 mg. of iron.

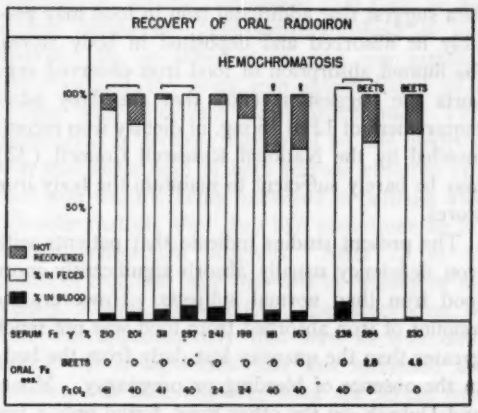

FIG. 7. RECOVERY OF RADIOIRON IN PATIENTS WITH HEMOCHROMATOSIS FOLLOWING ADMINISTRATION OF FERROUS CHLORIDE OR LABELED BEETS

These patients had not been venesected. The symbol ♀ identifies a female patient.

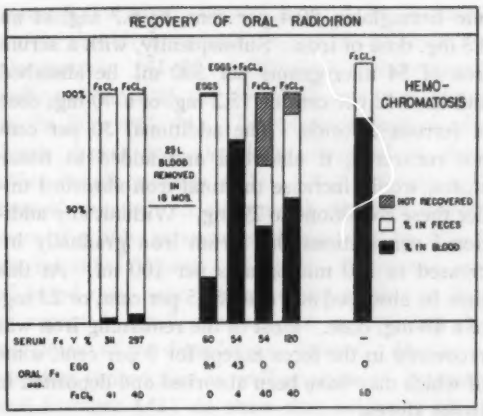

FIG. 8. RECOVERY OF RADIOIRON BEFORE AND AFTER INTENSIVE VENESECTION THERAPY OF A PATIENT WITH HEMOCHROMATOSIS (STUDIES 1 THROUGH 6)

Recovery was measured in a second patient (study 7) only after multiple venesections.

Absorption of Fe^{59} labeled eggs was not evaluated in any untreated patient with hemochromatosis. However, such a study was completed in one subject with hemochromatosis after the removal of 25 liters of blood over an 18-month period (Figure 8). Multiple iron absorption studies were completed before and after the venesection program. Initially the serum iron ranged between 235 and 311 micrograms per 100 ml. When studied at this stage he absorbed and incorporated into hemoglobin 4.7 to 6.2 per cent of a 40-mg. dose of ferrous chloride. Ninety-four per cent of the dose was recovered in stools. After extensive venesections, during which period his hemoglobin remained at essentially normal levels and approximately 13 gm. of iron were removed from his stores, his serum iron was reduced to 60 micrograms per 100 ml. At this time he absorbed and used 20.2 per cent of 9.4 mg. of food iron, equivalent to the absorption of 1.9 mg. of iron. This result approximates closely that obtained in subjects with iron deficiency who received labeled eggs. In contrast, when Fe^{59} was administered as ferrous chloride along with nonradioactive eggs, he absorbed and incorporated

into hemoglobin 80.4 per cent, or 6.7 mg. of an 8.3 mg. dose of iron. Subsequently, with a serum iron of 54 micrograms per 100 ml. he absorbed and used 42 per cent or 15.2 mg. of a 40-mg. dose of ferrous chloride. The additional 30 per cent not recovered, if absorbed and added to tissue stores, would increase the total iron absorbed under these conditions to 29 mg. Without any additional venesections the serum iron gradually increased to 120 micrograms per 100 ml. At this time he absorbed and used 54.5 per cent, or 22 mg. of a 40-mg. dose. Most of the remaining iron was recovered in the feces except for 9 per cent, some of which may have been absorbed and deposited in tissue stores.

A second patient with hemochromatosis was studied only after extensive venesections.[8] He received Fe^{59} without added carrier iron, and absorbed and utilized 88.5 per cent of the administered iron for the formation of hemoglobin. The remainder was recovered in stools.

In summary, neither beet iron nor the iron salt, ferrous chloride, is absorbed to any demonstrably greater extent by patients with well-established untreated hemochromatosis than by normal subjects. An intensive venesection program will significantly lower the serum iron and remove iron from the body stores. Under these circumstances, absorption of both ferrous chloride and egg iron is greatly increased, approximating that usually encountered in iron-deficient subjects.

DISCUSSION

Our studies indicate that normal subjects, iron-deficient patients, and patients with idiopathic hemochromatosis absorb ferrous chloride more readily than food iron. In many of these studies a solution of ferrous chloride was administered to the subject in a quantity that considerably exceeded the amount of iron usually eaten by an individual in a single average meal. Chemical or physical substances that might decrease iron absorption were excluded since the patients were fasting. In most cases a supplementary reducing agent such as ascorbic acid was given with the ferrous chloride. There is evidence that the administration of ascorbic acid may increase the absorption of ferrous iron salts (19). However, in the present study there was no apparent difference between the absorption of iron by 7 subjects[9] who did not receive supplementary ascorbic acid and comparable subjects who received this reducing agent with ferrous chloride. No systematic comparison was made of the absorption of ferrous iron salts administered to the same patient with and without a reducing agent.

Iron in considerable excess of the daily loss of 1 mg. may be absorbed when ferrous chloride is administered to normal subjects under the described experimental conditions. These results agree with those reported by Dubach, Callender, and Moore (27). Within the limitations imposed by inaccuracies in stool collection our data may be interpreted as lending support to their suggestion that normal subjects may absorb and deposit in tissue stores additional iron beyond that which is incorporated into hemoglobin.

Normal subjects absorbed much less iron after a single feeding of radioiron labeled eggs or vegetables. These observations were obtained under physiological conditions more closely approximating the ingestion of an average meal containing 5 mg. of iron. Only 0.3 mg. of iron at best, or approximately one-third of the daily iron requirement was absorbed and incorporated into hemoglobin. This observation agrees with previously reported studies (26). Although fecal recovery data suggest that additional iron in food may possibly be absorbed and deposited in body stores, the limited absorption of food iron observed supports the suggestion (26) that the daily adult requirement of 12 to 15 mg. of dietary iron recommended by the National Research Council (32) may be barely sufficient to maintain the body iron stores.

The present studies indicate that patients with iron deficiency usually absorb significantly more food iron than normal subjects. However, the amount of iron absorbed from food was not much greater than the quantity lost daily from the body in the absence of bleeding or pregnancy. Moore and Dubach, on the other hand, found only a few iron-deficient subjects who absorbed more food

[8] This subject was studied through the cooperation of Dr. Wayne Rundles, Duke University School of Medicine.

[9] These 7 subjects include 5 normal subjects, 1 iron-deficient patient, and 1 patient with idiopathic hemochromatosis.

iron than did normal individuals, although the coadministration of a reducing substance such as ascorbic acid significantly increased the quantity of iron absorbed (26). In our studies markedly limited absorption of food iron was observed in an iron-deficient patient with a subtotal gastrectomy. Iron as ferrous chloride was as readily absorbed by this patient as by the other iron-deficient patients. Partial gastrectomy may have influenced the absorption of food iron since there is some evidence that dietary iron is ionized, reduced to the ferrous form, and better absorbed at an acid pH (33–37).

The limited absorption of food iron in these studies suggests that it would be difficult for an iron-deficient patient to replenish his stores by diet alone. Supplementary medicinal iron seems indicated for individuals who undergo chronic blood loss, have increased physiological requirements, or have an absorptive defect due to an operative procedure, *e.g.*, subtotal gastrectomy, or steatorrhea (7, 26, 38, 39). Therapy should be continued after the hemoglobin level has been restored to normal since it has been shown that depleted iron stores are not easily reconstituted (40).

A different situation is observed in hemochromatosis. Although our studies indicate that neither ferrous chloride nor food iron is absorbed to any greater extent in well-established hemochromatosis than in normal subjects, it appears necessary to assume that excess iron must be absorbed during the developmental phase of this disease. By no other means can one satisfactorily explain the huge iron stores found in a patient with idiopathic hemochromatosis. This assumption is perhaps supported by our observation that absorption of both ferrous chloride and food iron was markedly increased in patients with hemochromatosis after they had undergone an intensive venesection program. After 12 to 15 grams of iron had been removed by weekly or biweekly 500-ml. venesections, the absorption of iron was approximately that seen in iron deficiency. It is significant that this increased absorption was observed even after the serum iron concentration had returned to normal (120 micrograms per 100 ml.).

Studies showing increased radioiron absorption in younger patients with hemochromatosis have been reported recently which lend support to the concept that iron absorption must be increased during the developmental stage of idiopathic hemochromatosis (41–43). External measurements indicated most of the absorbed iron was stored in the liver (43, 44). Increased absorption of a lesser degree has also been observed in three older female patients (43). Of 9 patients with hemochromatosis in our studies, the two women were the only patients who appeared to absorb an increased quantity of iron. Menstrual loss of blood may have had an effect similar to that of repeated, small venesections, so that full development of the disorder was delayed. Our studies after venesection as well as those of Peterson and Ettinger (41) do show that extensive blood loss will modify the absorption of iron even after hemochromatosis has fully developed. Increased absorption may then occur even with a normal serum iron concentration. These results suggest iron stores may reaccumulate in idiopathic hemochromatosis unless vensections are continued at intervals as recommended by Finch and Finch (45).

Although the present study has demonstrated greater absorption of iron salts than of food iron, it does not provide a clear explanation why this occurs. Multiple studies in normal subjects evaluating the comparative absorption of labeled egg iron and of ferrous chloride alone and with nonlabeled eggs suggest that the presence of egg will decrease the absorption of the iron salt. This decreased absorption may be related to the solid content of the test meal (46), or formation of an insoluble compound of iron with a chemical constituent of egg or bread. Hegsted, Finch, and Kinney (47) have shown that a high concentration of added phosphate will decrease absorption of iron by rats on a corn grit diet, presumably by formation of an insoluble iron phosphate. Similarly, soluble phytates may also interfere with iron absorption (46, 48). Conversely, rats on a corn grit diet with added iron will absorb large amounts of iron and produce progressive hemosiderosis of the tissues (49). Studies by Hegsted, Finch, and Kinney indicate that the low level of dietary phosphate attained with a corn grit diet was primarily responsible for increased absorption of iron (47). Such increased absorption of iron observed on a phosphate-deficient diet with excess iron might account for development of dietary hemosiderosis

observed in malnourished pellagrins in South Africa (50, 51).

The influence of phosphates on iron absorption may explain the present observations that egg iron was poorly absorbed and that addition of egg decreased absorption of ferrous chloride. Halkett, Peters, and Ross found that egg yolk iron is in the ferric state and is strongly complexed to the phosphate of yolk phosphoproteins (52). Formation of such an iron phosphate complex occurs both in the biological production of eggs and when iron is added to eggs *in vitro*. They further observed that egg yolk iron is not removed by peptic digestion and acidity unless a reducing agent is present. It appears egg iron is not readily available for absorption and it is not surprising that so little was absorbed in the present study as well as in previous animal studies (53). In contrast it has been shown that a 10 to 20 fold increase in the absorption of food (egg) iron generally occurs in iron-deficient subjects when large amounts of ascorbic acid are administered with the iron (7, 26). This effect is presumably dependent upon the reduction of iron to the ferrous form and may be accomplished by other reducing substances in food.

It is of interest to consider how results of the present studies may relate to the theory that the intestinal mucosa is an important regulator of iron absorption (24, 54–57). According to Granick (56), iron is transferred from the intestinal lumen to blood by a protein, apoferritin, present in cells of the intestinal mucosa. Iron is taken up by the mucosal cells until all apoferritin is converted to ferritin. No more iron may then be absorbed until ferritin has given up iron to plasma. This is the concept of the "mucosal block" originally suggested by Hahn, Bale, Ross, Balfour, and Whipple (24).

Dubach, Callender, and Moore (27) have presented evidence that this block is at best a partial one and that in certain conditions, such as refractory anemia, pernicious anemia in relapse, or hemolytic anemia, the mucosal block does not prevent iron from being absorbed in spite of adequate body iron stores. We have observed a similar increase in iron absorption in thalassemia minor and in renal anemia (44). Moreover, it would appear that the block must fail significantly during the developmental stage of idiopathic hemochromatosis and again in this disease after an extensive course of phlebotomies. Other examples of the alteration of the mucosal block in animals and men have been discussed previously in relation to factors that influence absorption of iron.

Finally, our data in normal subjects, as well as those of others (27) suggest that the "mucosal block" does not prevent absorption of an increased quantity of iron salt when it is administered in a single feeding to a fasting subject under optimal conditions, *i.e.*, as a solution of ferrous chloride with ascorbic acid, or when given in large quantities (4). Such excess iron absorption has also been observed after average doses of iron salts have been orally administered for a period of years (58, 59).

SUMMARY AND CONCLUSIONS

1. The iron salt, ferrous chloride, is absorbed far more readily and in greater quantity by normal subjects and by patients with deficient and excess iron stores than is iron present in certain foods (eggs, vegetables).

2. Egg and vegetable iron are not absorbed sufficiently to supply iron in the face of increased loss or increased physiological requirements.

3. Absorption of certain food iron and ferrous chloride in patients with well-established hemochromatosis is approximately equal to that observed in normal subjects. However, it can be assumed that excess quantities of iron must be absorbed during the developmental phase of this disease.

4. After removal of blood by multiple venesections absorption of ferrous chloride and egg iron by patients with idiopathic hemochromatosis is markedly increased.

5. Absorption of iron salts is significantly influenced by dietary factors which may modify the form and solubility of iron in the lumen of the gastrointestinal tract.

6. Further evidence has been presented to support previous data in the literature which indicated that the "mucosal block" to iron absorption is only relatively complete and may not uniformly prevent the excess accumulation of iron in the body.

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AN EVALUATION OF THE SINGLE INJECTION THIOSULFATE METHOD FOR THE MEASUREMENT OF EXTRACELLULAR WATER [1]

By LEON C. CHESLEY AND ABRAHAM LENOBEL

(From the Department of Obstetrics and Gynecology, State University of New York College of Medicine at New York City, and the Kings County Hospital, Brooklyn, N. Y.)

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The purpose of this paper is to examine the validity of the single injection thiosulfate method, proposed by Cardozo and Edelman (1) for the measurement of the extracellular fluid volume. The conclusion is that the method does not give a valid estimate of the extracellular fluid volume and probably does not even measure the volume of distribution potentially available to thiosulfate.

Cardozo and Edelman gave an accurately known amount of sodium thiosulfate (about 10 gm. of $Na_2S_2O_3 \cdot 5\ H_2O$ in 10 per cent solution) by intravenous injection over a period that varied from 7 to 16 minutes. They then collected venous blood samples at intervals and plotted the logarithms of the serum thiosulfate concentrations against time. The points usually fell on a straight line; this was extrapolated back to zero time, which arbitrarily was set as the moment the infusion had been started. If the points did not fall on a straight line, the test was discarded because of the variable clearance rate of the thiosulfate. The extrapolated value for serum concentration of thiosulfate (P_o) is presumed to be what the concentration would be if the thiosulfate were instantaneously injected and evenly distributed in its final volume of dilution. This extrapolated P_o is then divided into the total amount of thiosulfate injected to calculate the volume of distribution.

Aside from the fact that virtually none of the thiosulfate has been injected at this zero time, the method is based upon rather questionable assumptions, which will be discussed later.

In some of the evaluations of the single injection method that follow, comparisons were made between the volumes of distribution of thiosulfate and sucrose as calculated by different methods. The other procedures used were the infusion/slope method devised by Schwartz (2) and the calibrated infusion (IV minus UV) method of Deane, Schreiner, and Robertson (3).

MATERIAL AND METHODS

All experiments were done with hospitalized women. While they were not "normal," they were selected. Patients with any discernible cause for disturbance in hydration, such as fever, renal disease or heart disease, were excluded. All patients were less than age 50 years and most were in the third and fourth decades of life. Nearly all patients were fasting the day of the test. The few exceptions are those patients in whom simultaneous measurements were made of the volumes of distribution of sucrose and thiosulfate.

Reagent grade sodium thiosulfate ($Na_2S_2O_3$) was made up as a 6.7 per cent solution in either isotonic saline or 5 per cent dextrose. This gives the same thiosulfate concentration as Cardozo and Edelman used in their 10 per cent solutions of the hydrated salt. The solution was sterilized by Seitz filtration and given intravenously from a calibrated buret. The dose was roughly 0.5 ml. per pound of body weight and was given over a period of from 8 to 14 minutes. Collections of venous blood were begun 25 minutes after the end of the infusion and five samples were taken over the next 75 minutes. When the volume of distribution was measured by the infusion/slope method, the priming dose was followed by constant infusion of more of the same solution at the rate of 1.05 to 1.10 ml. per minute for periods of 2 to 3 hours. The constant infusion was given by a Bowman pump, which was recalibrated after each use.

The sucrose solutions, used in measuring the volume of sucrose distribution, were made up in 8 per cent concentration in either isotonic saline or 5 per cent dextrose. (The latter gives an appreciable blank in the chemical method for measuring sucrose).

In the calibrated infusion method this solution was given intravenously at the rate of 1.05 to 1.10 ml. per minute. Half-hourly collections of venous blood and of urine were begun 2 hours after the start of the infusion. After the collection of 3 or 4 blood and urine samples the thiosulfate was given for the single injection procedure.

In measuring the volume of sucrose distribution by the

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infusion/slope method, a priming dose of 8 per cent sucrose was followed by more of the same solution, as described for the administration of thiosulfate. Usually, simultaneous measurements were made of the volumes of sucrose and thiosulfate distribution, with sucrose and thiosulfate combined in the same solution. In the infusion/slope procedure, a minimum of three venous blood samples were taken during the constant infusion, the first being obtained about 90 minutes after the beginning of the infusion. Five more venous blood samples were collected over the 100-minute period following the end of the infusion. In both the single injection method and the infusion/slope method the test was discarded in any case where the logarithms of the serum thiosulfate or sucrose concentrations, plotted against time, were not closely fitted by a straight line. Also no calculations were made from the data obtained by the infusion/slope method unless the serum concentrations of thiosulfate and sucrose had attained constancy.

The solutions injected were hypertonic, but the volumes were small in comparison to the volumes of distribution, being about 1 per cent in the single injection procedure. In the constant infusion methods the rate of administration was 1.07 ml. per minute, so that in 3 hours the total volume given was of the order of 2 to 3 per cent of the volume of distribution. Meanwhile renal excretion and metabolism of the solutes (except for sodium chloride) kept pace with the infusion rate in the cases studied by the infusion/slope method. Furthermore, the procedure was the same in all experiments except that solutions were sometimes given in 0.9 per cent saline and sometimes in 5 per cent dextrose.

For chemical analyses, two samples of the infusion solution were diluted (1:1,000 for thiosulfate and 1:2,000 for sucrose); urine samples were suitably diluted (usually 1:500 or 1:1,000) and serum samples were prepared by precipitating the proteins. For thiosulfate the serum was diluted 5 times in the protein precipitation by tungstic acid and for sucrose it was diluted 10 or 20 times in precipitating the proteins by zinc hydroxide. All samples were analyzed in duplicate or quadruplicate. Thiosulfate was measured by the iodine titration method of Brun (4) and sucrose by the resorcinol method of Roe, Epstein, and Goldstein (5), using a Beckman DU spectrophotometer. Blood, urine, water and reagent blanks were analyzed with all measurements. When sucrose infusions were given in 5 per cent dextrose, an aliquot of the original dextrose solution was diluted 1:2,000, as was the infusion solution. These dextrose blanks were carried through the sucrose analyses and their optical densities were substracted from the optical densities of the infusion samples. This correction factor averaged about 4 per cent of the readings of the infusion samples.

RESULTS

Experimental alteration of the renal clearance rate

The first experiments with the single injection method were designed to find what effect changing the slope of the exponential disappearance rate of serum thiosulfate would have upon P_o and, therefore, upon the calculated volume of thiosulfate distribution. The slope of the exponential curve was changed in two ways: (*a*) by pre-medication with carinamide, which partially blocks the renal tubular excretion of thiosulfate (6), and (*b*) by intravenous theophylline ethylenediamine, which increases the glomerular filtration rate (7) and therefore the renal excretion rate of thiosulfate. More than half of the experiments could not be used because of the irregular effect of the drugs over the 100-minute period following the infusion of the thiosulfate. (This irregularity is reflected in a non-linear plot of the logarithms of the decreasing serum thiosulfate concentrations against time. Cardozo and Edelman wrote that such cases can not be used, for obvious reasons). By trial and error the best dose of carinamide was found to be 4 gm. given at 6:00 A.M. and 2 gm. given at 9:00 A.M., with the thiosulfate infusion given at 10:00 A.M. In the theophylline ethylenediamine experiments, 0.25 gm. of the drug was given intravenously about 10 minutes before the thiosulfate infusion and this was followed with a continuous infusion of another 0.25 gm. in 250

TABLE I

Experimental alteration of the clearance rate of serum thiosulfate by carinamide or theophylline, showing the times at which the experimental curves intersect with the curves found without medication and the percentage difference in calculated thiosulfate space resulting from the change in extrapolated P

Experiment:	1a	1b	2	3	4	5	6	7	8	9
Minutes after zero time at which curves cross:	29	30	2	No	18	21	27	29	16	36
Percentage difference in thiosulfate space resulting from changed excretion rate:	10	24	7	7	19	18	11	13	17	30

ml. of 5 per cent dextrose over the 100-minute period of the test.

Nine successful experiments were done in fasting women selected with special care as to "normality." In all cases the thiosulfate was given in 5 per cent dextrose. The order in which the experiments were done was varied from case to case and the tests were done on successive days in any one patient. All exponential disappearance curves for serum thiosulfate were fitted by the method of least squares to ensure objectivity. If the single injection method is valid, the disappearance curves should intersect at zero time, that is, P_o should be constant because the amount of thiosulfate given was constant and the potential volume of distribution should not vary much from test to test (see Discussion).

Table I shows that the disappearance curves did not intersect at zero time; in one case the lines did not cross at all and in the majority of cases the intersections occurred at 20 to 30 minutes after zero time. The experimental alteration of the renal clearance rate of thiosulfate changed the calculated volume of distribution in every case, the change being from 7 to 30 per cent. One of the experiments is shown in Figure 1, where the lines cross at 29 and 30 minutes. In this particular case, the thiosulfate space calculated from P_o with carinamide is 138 per cent of that calculated from P_o with theophylline ethylenediamine; the calculated volumes were 11.7 and 8.5 liters, respectively. This is the only experiment, in more than 20, in which three good disappearance curves were obtained. In the other eight cases reported there were two good curves, but the third with either carinamide or theophylline ethylenediamine departed from linearity on the semilog plot. Of the eight, five compare curves with no medication to curves with carinamide and three compare curves with no medication to those with theophylline ethylenediamine.

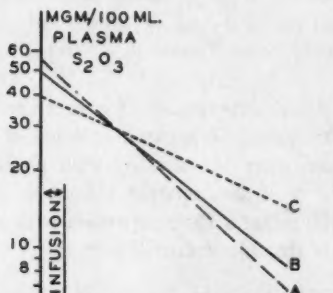

FIG. 1. THE EFFECT OF ALTERING THE RENAL EXCRETION OF THIOSULFATE UPON THE VALUE OF EXTRAPOLATED P_o

A = pre-medication with theophylline ethylenediamine, B = basal measurement with no medication, C = pre-medication with carinamide. Note that P_o is a function of the clearance rate of serum thiosulfate.

It would appear that the value for P_o is a function of the clearance rate of thiosulfate. Experimental alteration of the renal clearance rate changes P_o and results in changes in a predictable direction of the calculated volume of thiosulfate distribution. Decreasing the slope of the curve increases the calculated volume of distribution while increasing the slope of the curve decreases the calculated volume.

Comparison of thiosulfate spaces measured by the infusion/slope method to those calculated from the single injection method

The single injection method was compared with the infusion/slope method in six fasting patients, with alternation of the method first applied. In any one patient the tests were done on successive days. All solutions were given in 0.9 per cent saline.

The pairs of measurements did not check. In five of the six cases the volume of distribution for thiosulfate was considerably greater as measured by the infusion/slope method than as estimated from the single injection method. The results, in Table II, show that the average discrepancy was 30 per cent and ranged from 0 to 53 per cent.

Comparison of the thiosulfate space as estimated by the single injection method to the volume of sucrose distribution

In 18 patients the sucrose space was measured by the calibrated infusion method and immediately thereafter a single injection of sodium thiosulfate was given for the estimation of the volume of thiosulfate distribution. No experiment is included unless equilibrium of sucrose was established (that is, essentially constant volumes of

TABLE II

Comparison of measurements of the thiosulfate space by the methods of Cardozo and Edelman and of Schwartz and of the slopes of the serum thiosulfate disappearance curves in the two methods

Case:	1	2	3	4	5	6	Mean
Body weight, *Kg.*:	55.0	65.0	69.1	63.7	56.8	50.9	
Thiosulfate space							
Cardozo and Edelman:	9.8	7.8	7.9	8.2	8.8	10.6	8.85
Schwartz:	12.0	11.9	11.5	11.5	11.6	10.6	11.50
Ratio, Schwartz to Cardozo and Edelman methods:	1.22	1.53	1.46	1.40	1.32	1.00	1.30
Ratio, disappearance curve slopes, Cardozo and Edelman to Schwartz methods:	1.60	1.96	2.14	1.86	1.67	1.87	1.85

distribution in successive periods). In all but two cases the solutions were given in 5 per cent dextrose. The calculations from the data for the single injection method gave apparent volumes of distribution for thiosulfate that averaged about 35 per cent less than the sucrose space, as also was found by Ikkos (8). The apparent volume of thiosulfate distribution, as calculated by the single injection method, bore no constant relationship to the volume of distribution of sucrose, as the scattering of points in Figure 2 shows. Of course, sucrose may not measure the extracellular fluid volume but under the conditions of the experiments a steady state had been attained and a reproducible space had been measured. (This often was not

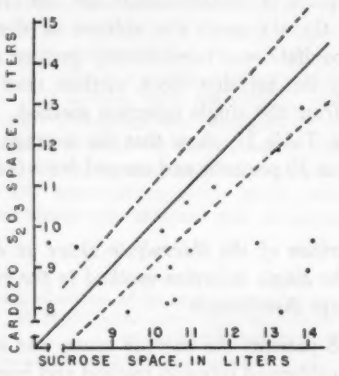

FIG. 2. COMPARISON OF THE THIOSULFATE SPACE, AS CALCULATED BY THE CARDOZO AND EDELMAN SINGLE INJECTION METHOD, TO THE SUCROSE SPACE MEASURED ALMOST SIMULTANEOUSLY

The solid line is the line of identity and the broken lines represent plus and minus 10 per cent.

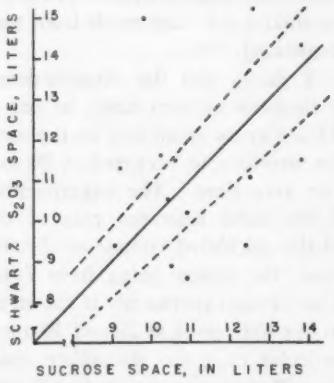

FIG. 3. COMPARISON OF THE THIOSULFATE SPACE, AS MEASURED BY THE SCHWARTZ INFUSION/SLOPE METHOD, TO THE SUCROSE SPACE MEASURED SIMULTANEOUSLY

The solid line is the line of identity and the broken lines represent plus and minus 10 per cent.

true in Ikkos' experiments, for in about 80 per cent of his cases the apparent volume of sucrose distribution increased steadily with time. Arbitrarily, he took the apparent volume of distribution at 150 minutes for comparison with apparent volumes of thiosulfate distribution.)

Comparison of the thiosulfate space measured by the infusion/slope method with the volume of distribution of sucrose

Measurements of the volume of distribution of thiosulfate, made with the infusion/slope method, were compared with simultaneous measurements of the volume of sucrose distribution in 14 patients. In nine of these the volume of sucrose distribution

TABLE III

Comparison of thiosulfate and sucrose spaces measured simultaneously by the infusion/slope method of Schwartz

Case:	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sucrose space, *l.*	10.4	13.7	9.0	10.9	10.6	12.5	10.2	10.5	13.8	12.5	10.9	9.2	12.4	9.8
Thiosulfate space, *l.*	10.5	14.3	9.4	10.2	11.5	11.1	11.6	8.9	11.5	15.1	8.6	11.3	9.4	15.1
Percentage deviation of thiosulfate space from sucrose space	1	4	5	6	8	11	14	15	16	21	21	23	24	54

was measured both by the infusion/slope method and by the calibrated infusion method; in the other five, one or the other of the methods was used.

Figure 3 shows that there was no consistent relationship between the thiosulfate and sucrose volumes of distribution, although the average difference was very small. Relative to the volume of sucrose distribution, the thiosulfate space varied from plus 54 to minus 24 per cent, as shown in Table III. While the average ratio is close to 1.0, the wide scatter of the points in Figure 3 indicates that this may be fortuitous.

Comparison of volumes of sucrose distribution measured simultaneously by the infusion/slope and calibrated infusion methods

Inasmuch as the calculations of the volume of thiosulfate distribution did not agree when the single injection and the infusion/slope methods were applied successively in individual patients, it is of interest to compare the infusion/slope method to some other procedure. Therefore simultaneous measurements of the volume of sucrose distribution were made by the infusion/slope and the calibrated infusion methods. Here the sucrose was given in 0.9 per cent saline and the priming dose of sucrose was added in the "IV" factor of the IV minus UV formula of the calibrated infusion method.

As Figure 4 shows, these methods, quite different in principle, gave very similar volumes for the sucrose distribution. All but 1 of the 10 pairs of measurements checked within 10 per cent. The points shown in the graph are symmetrically distributed about the line of identity and the average difference between the simultaneous measurements is zero. Here, the average ratio of 1.0 may not be fortuitous for, in contrast to the comparison of volumes of thiosulfate and sucrose distribution, the pairs of measurements check closely.

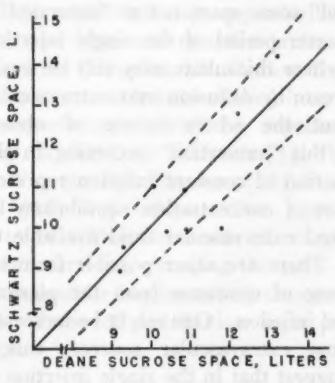

FIG. 4. COMPARISON OF SIMULTANEOUS MEASUREMENTS OF THE SUCROSE SPACE MADE BY THE SCHWARTZ INFUSION/SLOPE METHOD AND BY THE DEANE, SCHREINER, AND ROBERTSON CALIBRATED INFUSION (IV MINUS UV) METHOD

The solid line represents identity and the broken lines plus and minus 10 per cent.

DISCUSSION

The apparent advantage of the single injection method lies in its circumvention of the constant infusion and urine collections. However, its theoretic bases do not seem sound. Thiosulfate is known to enter the red blood cells (1) and if it enters these cells it may enter others. Gilman, Philips, and Koelle (9) found that in dogs about one-fourth of the injected thiosulfate cannot be recovered and they concluded, on good evidence, that most of the irretrievable fraction disappeared during and just after the infusion. The incomplete recovery was confirmed, for man, by Cardozo and Edelman. This together with the odor of the urine after thiosulfate injection suggest that

thiosulfate is metabolized in the body. Inasmuch as it is relatively stable in blood, *in vitro,* such metabolism probably occurs in cells.

The extrapolation of the decremental curve back to zero time implies questionable assumptions. One assumption is that thiosulfate disappears at the same exponential rate from the beginning of the infusion to the end of the blood collections (100 minutes). The assumed starting point for this exponential decrement in plasma concentration is based not upon the amount already injected at zero time, but upon the total amount to be injected over the next 10 or more minutes. This does not seem reasonable. Also, even when such injections are given within seconds, the early portion of the decremental curve is not adequately described by the course of the exponential limb, as shown by Sheppard, Overman, Wilde, and Sangren (10) and by Sapirstein, Buckley, and Ogden (11). Another assumption is shown graphically in the first figure in the paper by Cardozo and Edelman. The early portion of the decremental curve lies above the extrapolated exponential limb (as in all such cases); Cardozo and Edelman factor this early portion of the curve into two straight (exponential) lines by the standard procedure of subtracting the extrapolated limb from the observed points. From this they draw the conclusion that two processes are going on in the early minutes: (*a*) equilibration of the thiosulfate throughout plasma and interstitial fluid, and (*b*) clearance of thiosulfate at a uniform exponential rate, by renal excretion and metabolism. However, the rapid component of the decremental curve, in man, may represent the immediate loss of thiosulfate, which Gilman, Philips, and Koelle found in dogs. If the steep curve represents equilibration of thiosulfate throughout all of extracellular water, as Cardozo and Edelman suggest, the distribution must occur with almost incredible rapidity—within 10 minutes, as they say. In view of the time (30 to 120 minutes) required for sucrose to attain its maximum volume of distribution in normal subjects, it does not seem likely that thiosulfate would become evenly distributed in so short a time. The diffusion coefficients can not be specified exactly because of concentration effects. Cardozo and Edelman cited 0.68 cm.^{2} per day for thiosulfate and Raisz, Young, and Stinson (12) cited 0.55 cm.^{2} per day for sucrose; the source in both cases is the International Critical Tables.

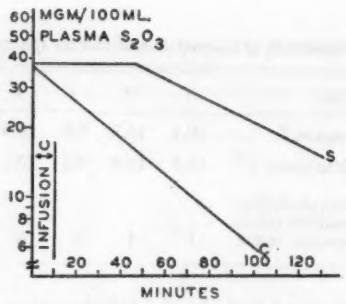

FIG. 5. COMPARATIVE RATES OF CLEARANCE OF SERUM THIOSULFATE IN THE SCHWARTZ INFUSION/SLOPE METHOD (S) AND CARDOZO AND EDELMAN SINGLE INJECTION METHOD (C)

The constant infusion of the Schwartz method had gone on for 2 hours before the beginning of the time shown in the graph, allowing time for the establishment of a steady state. "Infusion C" refers to the period of infusion in the Cardozo and Edelman method.

One observation is consistent with the interpretation that the steep component may represent immediate loss of thiosulfate. Figure 5 and the last line in Table II show that the slope of the serum thiosulfate disappearance curve was nearly twice as great in the single injection procedure as in the infusion/slope method. This suggests that the prolonged infusion of the latter method may have "saturated" some space not so "saturated" in the much shorter period of the single injection procedure, where thiosulfate may still be leaving the blood stream by diffusion into extravascular fluid throughout the whole course of observation. Possibly this "saturation" occurring in the prolonged period of constant infusion represents the attainment of concentration equilibrium between plasma and extravascular fluid available to thiosulfate. There are other possible factors in the slower rate of clearance from the plasma, after prolonged infusion. One might be increased feedback from extravascular sources; this, again, would suggest that in the single injection method the extravascular sources had not been brought into equilibrium with plasma water. Another possibility is that the rate of metabolism of thiosulfate slows down after prolonged infusion.

If a large proportion of the injected thiosulfate does disappear quickly, in man, then obviously the

dividend in the equation for the single injection method is too great and, of itself, should give calculated volumes of distribution that are too large. However, the volume calculated from this equation averages 30 per cent less than the measurements of the thiosulfate space by the infusion/slope method. This suggests that the divisor (P_o) in the equation for the single injection method is too large and in even greater error than is the dividend. This could result from the failure of thiosulfate to diffuse into and attain equilibrium in its potential total volume of distribution within the short period of time encompassed in the test. Thus, at least two variable and partially compensatory errors fortuitously result in calculations of an apparent thiosulfate space that often falls within the range described as normal for the volume of distribution of inulin.

The experimental alteration of the renal clearance of thiosulfate changes P_o and therefore the apparent volume of thiosulfate distribution as calculated in the single injection method. Since this work was completed, Peterson, O'Toole, and Kirkendall (13) have reported marked variations in the sucrose space of man, during a 24-hour period. Inasmuch as the present experiments, in which the renal clearance rate of thiosulfate was varied, were done on successive days, the assumption that the space should be nearly constant from day to day may be in error. In the three patients studied in regard to such reproducibility there was less than 5 per cent variation in the calculated volume of the thiosulfate distribution, from day to day. No more cases were studied because of the relative constancy reported for repeated estimations by Cardozo and Edelman (1), Ikkos (8), Raisz, Young, and Stinson (12) Becker and Joseph (14) and others. The reproducibility seems to be within the limits of analytic error. Even if it were not, changing the renal clearance rate of thiosulfate has invariably changed the calculated volume of distribution in a predictable direction.

SUMMARY

Experimental alteration of the renal excretion rate of thiosulfate, by pre-medication with carinamide or theophylline ethylenediamine, changed significantly the volume of thiosulfate distribution as calculated by the single injection method.

The volume of thiosulfate distribution, as measured by the infusion/slope method, averaged 30 per cent greater than the space calculated by the single injection method.

The thiosulfate space, measured by the infusion/slope method or calculated by the single injection method, bore no constant relation to the volume of sucrose distribution.

Simultaneous measurements of the sucrose space by the infusion/slope and by the calibrated infusion methods gave consistent results.

The failure of the single injection method to give thiosulfate spaces even approximating those measured by the infusion/slope method strongly suggests that the single injection method does not measure the volume of thiosulfate distribution.

It is concluded that the single injection method is not sound theoretically and that apparent volumes of distribution calculated from P_o have no significance as spaces. They only fortuitously come close to volumes measured by sucrose or inulin because of multiple, variable and partially compensatory errors.

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THE HEMODYNAMIC RESPONSE TO LANATOSIDE C OF DOGS WITH EXPERIMENTAL AORTIC-CAVAL FISTULAS

By OSCAR W. SHADLE,[1] THOMAS B. FERGUSON,[2] DAVID C. SABISTON, JR.,[3] AND DONALD E. GREGG

(From the Department of Cardiorespiratory Diseases, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.)

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Previous work has demonstrated that a severe burden is placed on the circulatory system of the dog by the surgical production of a long-sustained aortic-caval fistula (1). Circulatory distress is manifested by ascites, edema of all extremities and the lungs, together with a marked rise in left ventricular end-diastolic pressure, cardiac output, cardiac work, heart rate, and a depression of total peripheral resistance. The heart is at or near a state of cardiac decompensation, as indicated by the level of cardiac work and the response of the circulatory system to the infusion of whole blood or saline (1). Such infusions generally cause the cardiac output and cardiac work to decline and the left ventricular end-diastolic pressure to rise further, while the maximum level of cardiac work attained before infusion approximates that in the normal dog with massive infusion (1). To obtain further information on the physiological state of these dogs their response to rapid digitalization has been determined. This has been compared with the response of dogs without aortic-caval fistulas. The results of this study form the basis for this report.

METHODS AND PROCEDURES

A van Leersum carotid loop and cardiopexy were constructed first to aid in determining the arterial pressure, left ventricular end-diastolic pressure, and cardiac output (2). Later an anastomosis approximately 10 mm. in length was made between the aorta and inferior vena cava below the renal vessels (1). Weeks to months later measurements were made of the effect of Lanatoside C on the cardiac output, carotid blood pressure, heart rate, and left ventricular end-diastolic pressure. Pressures were recorded either by membrane manometers or by strain gauges connected to needles inserted into the left ventricular cavity and carotid loop. Figure 1 shows the character of the pressure curves obtained from the carotid loop and left ventricular cavity before and after digitalization of the anesthetized dog with an aortic-caval fistula. Cardiac output was calculated from the arterial dilution curve of Evans blue dye as modified for the recording cuvette densitometer (3). The dye was injected into the right atrium through a catheter inserted via the external jugular vein, and arterial blood was sampled from the carotid artery. Experiments were performed on the unanesthetized and anesthetized animal. The unanesthetized dogs had been trained previously to lie quietly on a table. In the anesthetized dogs anesthesia

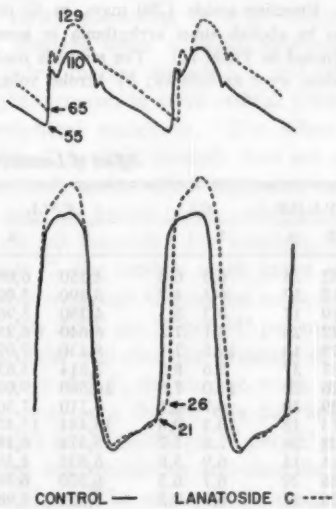

FIG. 1. SUPERIMPOSED TRACINGS OF CAROTID (UPPER) AND LEFT VENTRICULAR (LOWER) PRESSURE CURVES TAKEN BEFORE (SOLID LINE) AND 45 MINUTES AFTER (BROKEN LINE) DIGITALIZATION OF ANESTHETIZED DOG WITH AN AORTIC-CAVAL FISTULA

Numbers refer to carotid systolic and diastolic pressures and to left ventricular end-diastolic pressure in mm. Hg.

[1] Present address: Department of Physiology, University of Louisville School of Medicine, Louisville, Kentucky.

[2] Present address: Medical and Surgical Chest Service, Washington University School of Medicine, Saint Louis 10, Missouri.

[3] Present address: Investigator, Howard Hughes Medical Institute, Department of Surgery, The Johns Hopkins University and Hospital, Baltimore, Maryland.

TABLE I

Physical status of dogs with chronic aortic-caval fistulas

Dog No.	Weight (*Kg.*) Before	After	Fistula duration (*days*)	Remarks
1	13.1	12.6	37	No edema, ascites or evidence of circulatory stress
2	12.6	13.0	30	Hind leg edema. No ascites or evidence of congestive heart failure
3	15.5	21.4	55	Much edema of all extremities and ascites; tissue loss
4	11.8	11.2	49	Slight ascites and leg swelling
5	12.3	11.1	23	No ascites, edema or evidence of circulatory stress
6	10.0	13.8	40	Stormy history with marked ascites, edema of all extremities
7	17.3	15.4	385	Mild ascites and edema of front and hind extremities
		17.8	425	Marked ascites and edema of all legs with considerable recent weight gain
8	17.3	16.8	69	No ascites, slight edema
9	13.6	21.8	69	Considerable ascites and edema of front and hind legs. Pulmonary edema at death on 153rd day
10	14.6	14.1	70	Considerable edema of all legs
11	18.2	17.2	70	Pulmonary edema
12	17.3	20.9	150	Marked ascites, peripheral edema

was accomplished by premedication with 3 mgm. per Kg. of morphine sulfate, followed by 0.25 cc. per Kg. of a 1:1 mixture of Dial-urethane and pentobarbital. Arterial oxygen saturation was maintained by a demand valve apparatus connected to an oxygen tank; phasic intrapleural pressure was recorded with an optical segment capsule connected to a trocar in the left intrapleural space near the heart, and these pressures were used to correct the simultaneously recorded left ventricular end-diastolic pressure. Procaine amide (200 mgm. in 50 cc. saline) was given to abolish sinus arrhythmia in some of the animals (noted in Table II). The methods used to analyze the data were as follows: 1) Stroke volume index in cc. per M^2 (stroke volume/surface area M^2); 2) stroke work index in gm.M per M^2 (stroke volume index × (mean arterial pressure − left ventricular end-diastolic pressure) × 13.6); 3) cardiac work index in gm.M per M^2 per min. (cardiac index × (mean arterial pressure − left ventricular end-diastolic pressure) × 13.6); and 4) total peripheral resistance (mean arterial pressure × 100/cardiac output in cc.).

After control observations of cardiac output and pressures had been completed, either 0.045 or 0.025 mgm. per Kg. of Lanatoside C was given intravenously as a single dose and at the end of 30 to 60 minutes the observations were repeated. The larger amount of the drug is at

TABLE II

Effect of Lanatoside C in dogs with aortic-caval fistulas †

Dog No.	L.V.E.D.P. B	A	C.I. B	A	C.W.I. B	A	S.V.I. B	A	S.W.I. B	A	M.A.P. B	A	T.P.R. B	A	H.R. B	A
1	32	35	6.5	6.8	4,150	6,080	62	60	40	54	79	101	2.1	2.5	104	113
2	15	12	4.3	4.7	3,390	5,000	59	55	45	59	71	90	2.8	3.3	74	85
3	19	17	4.3	5.1	4,250	5,960	34	39	33	45	91	103	2.7	2.6	129	132
4	22	26	8.1	7.2	6,640	6,250	66	68	54	59	82	90	1.8	2.2	122	106
5	19	13	10.5	7.9	8,430	7,070	69	53	55	47	78	79	1.4	1.9	152	150
6	37	37	9.6	8.8	2,814	3,638	58	56	17	24	58	67	1.0	1.3	167	157
7	26	22	11.0	7.6	10,580	9,040	76	77	73	91	97	109	1.4	2.2	145	99
7′	29	30	6.7	6.1	6,710	7,503	69	63	69	77	103	120	2.2	2.7	97	97
8	13	15	10.1	9.6	15,444	13,474	94	90	143	124	125	117	1.8	1.8	108	108
9*	28	26	7.6	7.7	5,374	6,180	55	59	40	48	80	85	1.3	0.9	138	130
10*	14	14	6.9	5.8	6,832	4,318	86	71	86	54	87	70	2.0	3.1	80	80
11*	28	29	6.7	6.5	6,370	6,365	39	44	38	42	98	102	1.7	2.2	170	150
12	26	17	6.9	8.3	5,161	5,983	50	69	37	49	81	70	1.4	1.0	138	120
Aver.	24	23	7.6	7.1	6,620	6,682	63	62	56	60	87	93	1.8	2.1	125	117

† Measured and calculated parameters *before* (B) and 30 to 60 minutes *after* (A) the injection of Lanatoside C. Left ventricular end-diastolic pressure, mm. Hg (L.V.E.D.P.); cardiac index (C.I.); cardiac work index (C.W.I.); stroke volume index (S.V.I.); stroke work index (S.W.I.); mean arterial pressure, mm. Hg (M.A.P.); total peripheral resistance (T.P.R.); and heart rate (H.R.). The data under 7 and 7′ were obtained from the same animal on the 385th and 425th post-operative day. The asterisk indicates those dogs receiving 0.025 mgm. per Kg. of Lanatoside C; all others received 0.045 mgm. per Kg. Animals 1 to 7 were studied under Dial-urethane pentobarbital anesthesia and received procaine-amide to abolish sinus arrhythmia. Animals 8 to 12 were trained and determinations were made using local procaine (1 per cent) subcutaneously at the site of needle puncture.

TABLE III

Effect of maintenance digitalization in dogs with aortic-caval fistulas *

Dog No.	Weight (Kg.)		L.V.E.D.P.		C.I.		C.W.I.		S.V.I.		S.W.I.		M.A.P.		T.P.R.		H.R.	
	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
8	16.8	16.8	13	4	10.1	6.7	15,444	7,470	94	61	143	68	125	86	1.8	1.8	108	110
9	21.8	19.5	29	21	7.6	7.1	5,374	8,015	55	44	40	50	80	104	1.3	1.8	138	160
10	14.1	13.6	14	9	6.9	4.5	6,832	4,957	86	45	86	49	87	89	2.0	3.5	80	100
11	17.2	17.2	28	32	6.7	6.3	6,370	4,660	39	45	38	34	98	86	1.7	1.9	170	140
12	20.9	20.9	26	33	6.9	7.9	5,161	3,700	50	66	37	31	80	68	1.4	0.9	138	120
Aver.	18.2	17.6	22	20	7.6	6.5	7,836	5,760	65	52	69	46	94	87	1.6	2.0	127	126

* Animals 8, 9, 10, and 11 maintained on Digitoxin for four days after initial digitalization with Lanatoside C; studies made before and on fourth day after digitalization. Animal 12 studied before and one day after digitalization. Abbreviations as in Table II.

least 30 per cent of the lethal dose reported for some other cardiac glycosides (4), and is about twice the dose used per kilogram of body weight in the studies by Stead, Warren, and Brannon (5) on human subjects in congestive heart failure. This amount of the drug is an effective dose, as indicated by the work of Li and Van Dyke (6). In one unanesthetized animal, the observations were repeated the next day and in four others, they were repeated after four days of digitalization. The four animals received daily maintenance doses of digitoxin, administered intramuscularly in amounts equivalent to one-tenth of the original digitalizing dose of Lanatoside C (Table III).

RESULTS

In the group of dogs with fistulas hemodynamic studies were made 23 to 425 days after creation of the aortic-caval fistula. The condition of the dogs and their hemodynamic status varied considerably (Table I). Some gained a considerable amount of weight with ascites and edema of all extremities while others gave no external evidence of circulatory stress or insufficiency. Table II presents the data obtained in the group with fistulas, both anesthetized (1–7) and unanesthetized (8–12), before and 30 to 60 minutes after the intravenous administration of Lanatoside C. It can be seen that before digitalization the average work load of these hearts was quite high. The left ventricle maintained an average cardiac index of 7.6 liters per minute, a cardiac work index of 6,620 gm. meters, and a stroke volume index of 63 cc. The carotid pulse pressure averaged 60 mm. Hg (data not shown), despite a heart rate of 125 per minute and a high left ventricular end-diastolic pressure of 24 mm. Hg. In some dogs previous measurements indicated that the extent of cardiac work response, after reaching a high level, was maintained. In others, maximum cardiac performance had been passed at the time of these studies. For example, in dog 7 after induction of the fistula the cardiac index dropped from 11 liters on the 385th day to 6.7 liters on the 425th day. During the interim the dog developed massive edema of the extremities, ascites, dyspnea, and increased body weight.

Although following digitalization, the dogs with fistulas varied somewhat in their response, the average changes show a slight decrease in the cardiac index, cardiac work index, and heart rate, with slight increases in mean arterial pressure and total peripheral resistance. The effect of the Lanatoside C in these animals does not correlate with the loads under which the heart was working, or with the height of the end-diastolic pressure in the left ventricle. For example, following Lanatoside C, the cardiac work index of dog 2 increased even though the cardiac work index was initially only 3,390 gm.M per M^2 per min. with a left ventricular end-diastolic pressure of 15 mm. Hg, while in dog 7, the cardiac work index fell after the drug even though this dog had a high cardiac work level (10,580 gm.M per M^2 per min.) and a high left ventricular end-diastolic pressure (26 mm. Hg). The effect of the drug on these dogs appears independent of the hemodynamic state of the circulation or of the work load on the heart.

Table III presents the data from five dogs with fistulas that were maintained on digitoxin from 1 to 4 days after initial digitalization with Lanatoside C. Their hemodynamic status was not improved.

TABLE IV

Effect of Lanatoside C in normal dogs *

Dog No.	L.V.E.D.P.		C.I.		C.W.I.		S.V.I.		S.W.I.		M.A.P.		T.P.R.		H.R.	
	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
1	9	9	1.8	1.6	1,870	2,000	36	34	36	44	86	103	8.0	11.0	49	46
2	8	8	1.9	2.4	2,500	3,890	29	29	37	47	103	128	9.3	9.3	68	83
3	17	11	2.9	2.1	3,290	2,370	52	48	59	55	100	95	6.4	8.6	56	43
4	7	5	1.7	1.4	1,735	1,440	29	27	29	27	81	79	9.2	10.9	59	53
5	12	12	3.0	2.8	3,080	3,890	40	48	42	65	88	114	5.1	7.0	74	59
6	15	12	2.6	2.0	2,370	1,970	26	24	24	24	83	86	6.2	8.4	101	81
7a	7	7	2.6	2.4	3,610	2,860	43	45	60	53	109	95	8.2	7.5	60	54
b		7		2.4		3,650		40		61		119		9.7		60
Aver.	10	9	2.4	2.1	2,640	2,760	37	37	41	47	93	102	7.5	9.0	67	60

* Animals 1, 2, 3, 4, 5, and 6 anesthetized. Animal 7 trained, unanesthetized; 7a and b data obtained 30 and 60 minutes, respectively, after digitalization. Abbreviations as in Table II.

Actually, four of the dogs showed a considerable reduction in cardiac work.

The influence of Lanatoside C upon the circulation was assessed in seven dogs without aortic-caval fistulas (Table IV). Six were anesthetized and one was trained and unanesthetized. The data in Table IV indicate that the performance of the left ventricle was not greatly altered by Lanatoside C. The average changes following rapid digitalization are very similar to those seen in the animals with fistulas. A slight decrease in cardiac index and heart rate with an increase in mean arterial pressure and peripheral resistance was noted.

DISCUSSION

The results of this study are in agreement with previous reports in the literature which indicate that in the normal heart digitalis, when effective, generally decreases cardiac output and heart rate and increases mean arterial pressure (7, 8).

In the animals with fistulas it is difficult to be certain that heart failure was always present since the direct effects of the fistula would be to produce hind-limb edema, increase venous pressure, and perhaps cause ascites as well. However, there were other physical signs and physiological measurements which indicated that these animals were at or beyond their maximum cardiac work level. Some animals developed edema of the fore-limbs as well as the hind-limbs, and a number of the dogs died of pulmonary edema which is highly indicative of cardiac failure under these circumstances. The maximum cardiac work levels in these preparations were similar to those obtained in normal dogs with massive infusion of blood or saline (1). The left ventricular end-diastolic pressure was consistently elevated in the fistula group, the lowest value being 13 mm. Hg with an average value of 24 mm. Hg; whereas, the corresponding value for the normal animals was 10.7 mm. Hg. Furthermore, the infusion of whole blood or saline into these animals led to an additional large increase in left ventricular end-diastolic pressure, and an early decline in cardiac work and output (1). This evidence indicates that these dogs were working at or very near the limit of their cardiac performance.

Acute digitalization with Lanatoside C failed to improve the cardiac work response in animals with aortic-caval fistulas. The cardiac index did increase in four of the twelve dogs, but the increase was only minimal and one of the animals had shown a similar increase following digitalization before the aortic-caval fistula had been constructed. Four of the animals were maintained on digitalis with no improvement, confirmatory evidence that digitalization did not improve cardiac performance. These data do not explain the lack of response to digitalis in these animals, but they do indicate that there is an undefined difference between these animals and, for example, animals with cardiac failure produced by pulmonary artery constriction (9).

SUMMARY

Dogs were prepared with a chronic aortic-caval fistula. At the time of study, performance of the left ventricle was characterized by a high level of

left ventricular end-diastolic pressure, cardiac output, stroke volume, and stroke work, and in most instances with one or more evidences indicative of cardiac failure such as pulmonary edema, edema of the extremities, and ascites. After a period varying from one to 14 months, the dogs were digitalized rapidly with Lanatoside C and the hemodynamic effects were observed during the next hour and in some animals after four days of maintenance dosage on digitoxin. Measurements of various cardio-dynamic parameters indicated that digitoxin did not alter measurably the cardiac performance.

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THE DISTRIBUTION OF SODIUM AND CHLORIDE AND THE EXTRACELLULAR FLUID VOLUME IN THE RAT[1]

By DONALD B. CHEEK, CLARK D. WEST, AND CATHERINE CARTER GOLDEN

(From The Children's Hospital Research Foundation and Department of Pediatrics, University of Cincinnati, Cincinnati, O.)

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Our attempts to gain information concerning the amount of electrolyte in cell water and the theoretical concentration of intracellular cations necessarily revolve around our ability to partition total body water into extracellular and intracellular phases. The total body potassium, for example, is confined almost exclusively to the intracellular phase, and if the intracellular volume of the body could be defined, the net intracellular potassium concentration could be readily calculated as:

$$\frac{\text{Total body K} - \text{Extracellular K}}{\text{Intracellular fluid volume}}.$$

The most rational interpretation of extracellular fluid volume would seem to be that defined originally by Manery, Danielson, and Hastings (1), by Manery and Hastings (2) and later by Nichols, Nichols, Weil, and Wallace (3). These authors divide the extracellular fluid into the following components: the plasma water, the interstitial water, and the connective tissue water. The interstitial water is considered that volume of fluid which rapidly equilibrates with substances such as inulin. Connective tissue water is the volume of water which is principally associated with collagen and elastin, and with which substances such as inulin equilibrate slowly. The study of Nichols, Nichols, Weil, and Wallace (3) would indicate that the sum of these three phases of the extracellular fluid is defined reasonably well by the chloride space if correction of the chloride space is made for intracellular chloride, and for the slightly greater concentration of chloride in connective tissue water than is present in an ultrafiltrate of serum.

The major aim of the present study is to test further the validity of the chloride space, corrected for the factors described above, as a measure of the extracellular volume by demonstrating an identity between it and the space calculated from the sodium content of extracellular fluid. Extracellular fluid volume has been calculated from sodium by the expression (extracellular sodium)/$(Na)_{ef}$ where extracellular sodium is the difference between total body sodium and the sodium which is not in the extracellular fluid (*i.e.*, that in bone, cells, and within the lumen of the gastrointestinal tract) and $(Na)_{ef}$ is the sodium concentration of the serum ultrafiltrate.

Values for total body chloride and sodium on which the calculations depend have been obtained by carcass analysis of a large number of normal rats (4, 5). Values for bone sodium have been calculated from the Na/Ca ratio for bone and from total body calcium. Values for cell sodium in muscle have been taken from data in the literature (6) calculated on the assumption that chloride in muscle occupies the same space as inulin and an exclusively extracellular position (7). Values for sodium in red cells were also calculated from the data of other investigators (8, 9) while those in visceral cells have been assessed by comparison of the volumes of distribution of inulin and sodium in the viscera following a constant infusion of inulin according to the technique described by Cotlove (7).

The good agreement between the sodium and chloride spaces calculated by correcting the value for total body electrolyte in this manner lends credence to the view that a value closely approximating the true extracellular volume of the rat has been determined. On this assumption the data obtained by carcass analysis have been used for further calculation of the intracellular volume and the theoretical concentrations of electrolyte within the cells.

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METHODS AND CALCULATIONS

The animals used in the study were male Wistar rats of the Hamilton Farms strain. For convenience and simplicity, a rat with a fat free dry solid content of 50 grams has been taken as a representative animal. Assuming a normal body fat content, such an animal would weigh approximately 220 grams.

Total body electrolyte and water. The carcass electrolyte and water content of the hypothetical 220-gm. rat was determined from regression equations calculated from data obtained by carcass analysis of 30 to 40 rats of normal health and vigor. The weight range of the animals was from 70 to 410 gm. with the greatest number falling around 220 grams (4, 5). The animals had been under observation at least two weeks prior to sacrifice and for ten days had been on a low residue diet of composition previously described (4). The regressions of total body electrolyte, water and nitrogen on fat free dry solid are given in Table I.

The methods of carcass analysis, with the exception of that for potassium, have been given previously (4, 5). In previous studies, potassium was determined on an acid extract of ashed carcass by flame photometry, while in the present study potassium in the ash has also been determined with chloroplatinic acid by the method of Consolazio and Talbott (10) modified to determine the salt K_2PtCl_6 gravimetrically instead of by iodometric titration. The method is to be published later in detail (11). The flame photometric method, employed on 47 animals, yielded a regression equation with a lower slope (0.283), a higher intercept (+ 0.36) and a greater scatter of the data (standard error of the estimate, 1.08 mEq.) than the gravimetric procedure. For animals of a fat free dry solid content of 50 gm. with which the present paper is concerned, the two equations gave almost identical values. By the gravimetric procedure, carcass potassium would be 14.38 mEq. as compared with 14.51 by flame photometry.

Estimation of bone sodium. Bone sodium was estimated in five healthy male rats of 210 to 230-gm. weight. These animals were of the same strain and prepared in the same manner as those used for carcass analysis. After sacrifice and exsanguination, the carcass was dried at 100 to 105°C as previously described. After drying, the larger bones could be easily removed by pulling away the dried tissues and cutting free adherent cartilage. The bones were broken into small pieces, the fat extracted in a Soxhlet apparatus and the pieces further titrated to fine particles in a mortar. A portion of the particles were then ashed in a platinum crucible and the ash dissolved in 5.0 ml. of weak HCl. Separate aliquots were taken for calcium determination by permanganate titration and for sodium determination by the gravimetric method of Butler and Tuthill (12). A separate aliquot of the bone particles was subjected to alkaline ashing and analyzed for chloride by the microdiffusion technique. The methods are identical to those used for carcass and are described in greater detail in previous papers (4, 5). The difficulties inherent in the flame photometric determination of bone sodium through interference by calcium (13) are obviated by use of the gravimetric procedure.

The sodium incorporated in bone salt was determined by subtracting from total bone sodium the amount of sodium calculated as present in the chloride space of bone. The total bone salt sodium of the carcass was calculated as total bone salt sodium

$$= \frac{\text{bone salt sodium (mEq./100 gm. FFDS)}}{\text{bone calcium (mEq./100 gm. FFDS)}} \times \text{total body calcium (mEq.)}.$$

Determination of total body collagen and of connective tissue chloride. Determinations of carcass collagen were performed on nine rats weighing approximately 220 gm., prepared before sacrifice in the same manner as those used for carcass analysis. The method of collagen estimation was that of Spencer, Morgulis, and Wilder (14). A homogeneous aliquot of powdered, fat free, dry carcass was autoclaved to convert collagen to gelatin, the gelatin was extracted with hot water and finally precipitated with tannic acid.

The chloride concentration of connective tissue water was determined by analysis of the achilles tendon of a normal rat and of a normal female mongrel dog. After exposure of the tendon, dissection was carried out rapidly, and each piece removed was immediately placed in a

TABLE I

Body composition in the normal rat (wt. range 70 to 410 grams): Regressions of total body electrolyte, nitrogen and water on fat free dry solid

Regression equations	Standard error of estimate	Number of rats	Values for 220-gm. rat (FFDS = 50 gm.)
Na_t (mEq.) = 0.193 FFDS + 0.78	± 0.64 mEq.	36	10.43 mEq.
Cl_t (mEq.) = 0.131 FFDS + 1.04	± 0.41 mEq.	35	6.73 mEq.
K_t (mEq.) = 0.323 FFDS − 1.77	± 0.75 mEq.	29	14.38 mEq.
Mg_t (mEq.) = 0.1014 FFDS + 0.594	± 0.32 mEq.	32	5.66 mEq.
Ca_t (mEq.) = 2.200 FFDS − 1.74	±11.50 mEq.	35	111.70 mEq.
P_t (mM) = 0.849 FFDS + 0.300	± 3.20 mM	30	42.75 mM
N_t (grams) = 0.131 FFDS − 0.065	± 0.200 gm.	40	6.49 gm.
H_2O_t (ml.) = 2.725 FFDS + 13.19	± 3.70 ml.	36	149.40 ml.
Average fat content: 9.6% body wt. Range 15.3 to 3.3%		36	

separate tared stoppered weighing bottle to minimize evaporation of water. After weighing, the tendon was dried to constant weight at 100 to 105°C, reweighed, ground in a mortar and fat extracted. Analyses for chloride, collagen and total nitrogen were carried out as for carcass (4, 5, 14).

Determination of extracellular sodium and chloride of viscera. Using inulin as a reference substance, sodium and chloride were partitioned between extra and intracellular compartments in the lungs, trachea, esophagus, stomach, intestines and testes by the method described by Cotlove for muscle (7). The data were obtained on 11 male rats whose weights ranged from 290 to 310 gm. The animals were placed on a low residue diet (4) four days before experimentation and fasted for 24 hours before sacrifice. Under ether anesthesia either a polyethylene catheter or a 22 gauge needle was placed in a tail vein for inulin infusion and the animal confined in the apparatus described by Kellogg, Burack, and Isselbacher (15). Following priming with an amount of inulin calculated as sufficient to raise the plasma inulin concentration to 300 mg. per cent, an infusion of inulin was maintained at a constant rate using an infusion machine. Both priming and maintenance solutions of inulin contained 7.5 gm. of inulin and 0.64 gm. of sodium chloride per 100 ml. After 6 hours of infusion in 7 animals and after 24 hours in 4 animals, ether anesthesia was again applied and the animal exsanguinated by aortic puncture. The infusion was stopped at the moment of aortic puncture. The lungs, gastrointestinal tract and testes were then quickly removed and placed in a tared vessel and weighed. Forty milliliters of water were then added and the tissues homogenized in a micro Waring blendor. The homogenate was placed in a boiling water bath for 30 minutes and then filtered. While in the water bath, the tubes were covered with aluminum foil to reduce evaporation. Serum and filtrate were analyzed for sodium by flame photometry and for inulin by the method of Schreiner (16) after preliminary hydrolysis with alkali as described by Ross and Mokotoff (17). Chloride in the tissue filtrate was determined by the micro diffusion technique of Conway (4, 18) and serum chloride by the method of Van Slyke (19). In early experiments the liver was analyzed separately, but the data are not included in the presentation because, after 24 hours of infusion, the inulin space was found to be larger than either the sodium or chloride space.

Inulinoid blanks were determined on the serum and filtrate of the visceral organs of 13 rats which received no inulin. These animals had been on the same diet as the experimental animals for four days before sacrifice and fasted for 24 hours before sacrifice. The inulinoid blanks for serum after alkali hydrolysis were found to be negligible and were disregarded. Blank values for the filtrate of viscera ranged from 2.4 to 4.7 mg. per cent. The average value was 3.44 and the standard deviation ± 0.52 mg. per cent. Inulin concentrations in the viscera filtrates of rats infused with inulin ranged from 25.4 to 56.8 mg. per cent with an average value of 38.0 mg. per cent. Serum inulin levels averaged 290 with a range from 209 to 428 mg. per cent. The total inulin and electrolyte content of the tissue was calculated as the product of viscera filtrate concentration times the volume of added water plus tissue water. Tissue water was taken as 81 per cent of wet weight (20).

The sodium and chloride spaces in viscera were corrected for the quantities of sodium or chloride present in the lumen of the gastrointestinal tract. The amount of the correction was determined by analysis of the luminal contents of five rats which had been on a low residue diet for four days and fasted for 24 hours before sacrifice. No infusion was given. After anesthetizing with ether, the lower end of the gastrointestinal tract was clamped, the gut dissected free and placed in a beaker. A rubber catheter was then inserted in the cardia of the stomach and 50 ml. of 5 per cent glucose in water rapidly injected so that the full length of the gut was distended. The clamp was then removed and the fluid gently pressed out through several incisions made in the gut wall. The whole procedure required only a few minutes. The volume of fluid was then measured and, after filtering, analyzed for sodium and chloride. The volume of fluid recovered averaged 49.6 ml.

Estimation of gastrointestinal fluid volume. The values for total body water determined by carcass analysis were corrected for the water contained within the gastrointestinal tract. The volume of fluid within the gut lumen was determined in six rats weighing approximately 220 grams. The animals were prepared in a manner identical to those subjected to carcass analysis; a low residue diet was fed for five days with free access to water. They were then sacrificed after a four-hour fast at the same time of day as the animals used for carcass analysis. The gastrointestinal tract was dissected free, placed on a board and opened longitudinally with scissors. The gut surface was then gently swabbed with gauze which had been previously dried by heating at 100° C. The gauze was immediately placed in a weighing bottle, covered and weighed. The bottles were then uncovered, placed in an oven at 100° C for 24 hours and re-weighed. The water content of the gut in milliliters was taken as the weight difference of the gauze in grams.

Calculation of sodium, chloride, and inulin spaces. The volumes of distribution of sodium and chloride were calculated by dividing the amount of the ion present by the concentration in a serum ultrafiltrate. To determine serum ultrafiltrate concentration, the serum concentration was corrected for serum water and Donnan equilibrium as follows:

$$(Na)_{uf} = \frac{(Na)_s}{0.93} \times 0.95$$

and

$$(Cl)_{uf} = \frac{(Cl)_s}{0.93} \times \frac{1}{0.95}$$

in which $(Na)_{uf}$ and $(Cl)_{uf}$ are the amounts of sodium and chloride per liter of serum ultrafiltrate, $(Na)_s$ and $(Cl)_s$ the amounts per liter of serum, and 0.95 the Don-

nan factor. Serum water was taken as 930 ml. per liter of serum and is represented by the factor 0.93.

Serum ultrafiltrate concentrations of inulin were calculated as $(In)_s/0.93$ where 0.93 is again the serum water correction.

RESULTS

Calculation of extracellular volume from extracellular chloride

The total body chloride of a 220-gram rat and the corrections necessary for the estimation of that portion of total chloride which is in extracellular fluid and at a concentration equal to that in a serum ultrafiltrate are given in Table II. A description of the calculation of these corrections follows.

Correction for connective tissue chloride. To correct total body chloride for the chloride in connective tissue in excess of that predicted from the chloride concentration of the serum ultrafiltrate, analyses of dog and rat tendon for water, chloride, collagen and total nitrogen were carried out. The data are given in Table III. In both species, the actual chloride content of tendon was 0.76 mEq. per 100 gm. of FFDS greater than would be predicted from the tendon water content and the serum ultrafiltrate chloride concentration. In terms

TABLE II

Calculation of extracellular fluid volume from extracellular chloride in a 220-gram rat (50 gram FFDS)

	mEq	Percent of Total
Chloride Outside Extracellular Fluid		
Chloride of connective tissue in excess of predicted (from determination of "excess" chloride in rat and dog tendon and from rat total body collagen)	0.10	1.5
Erythrocyte chloride (from chloride concentration of red cell water (8), blood volume and hematocrit (9), and red cell water content)	0.41	6.1
Chloride intracellular in muscle (7)	0	0
Chloride intracellular in liver (from difference between sodium and chloride spaces of liver)	0.06	0.9
Chloride within lumen of gut (determined by flushing of gut)	0.16	2.4
Chloride intracellular in viscera, exclusive of liver, brain, spleen, and kidneys (from comparison of sodium, chloride and inulin spaces)	0.12	1.8
Total	0.85	12.7
Total Body Chloride	6.73	
Chloride in Extracellular Fluid	5.88	87.3

Serum ultrafiltrate chloride concentration, 124 mEq/l.

Chloride space = $\frac{5.88}{.124}$ = 47.4 ml.

TABLE III

Chloride and water content of tendon in dog and rat: Calculation of "excess" chloride in tendon

	(1) Tendon water *ml./100 gm. FFDS*	(2) $(Cl)_{sf}$* *mEq./L.*	(3) Tendon Cl predicted (1) $\times \frac{(2)}{1,000}$ *mEq./100 gm. FFDS*	(4) Tendon Cl found *mEq./100 gm. FFDS*	(5) "Excess" chloride (4)−(3) *mEq./100 gm. FFDS*
Dog	128	127	16.26	17.02	0.76
Rat	140	125	17.50	18.26	0.76

* $(Cl)_{sf}$ = Chloride concentration of serum ultrafiltrate.

of tendon water content, tendon chloride amounted to 133 mEq. per L. in the dog and 131 mEq. per L. in the rat; in both species the values were 6 mEq. per L. greater than the concentration of the serum ultrafiltrate.

Taking the excess chloride in connective tissue as 0.76 mEq. per 100 gm. of FFDS, the excess chloride associated with collagen in the whole rat may be calculated as 0.76/100 × total body collagen (grams). Total body collagen in nine rats of approximately 220 grams body weight averaged 11.9 ± 0.48 (SD) grams. The excess chloride associated with connective tissue then becomes 0.10 mEq. The calculation assumes that all body collagen has the same affinity for chloride as does achilles tendon. No correction has been made for the fact that achilles tendon is not pure collagen; by analysis only 91 per cent of the total nitrogen of the tendon could be accounted for as collagen nitrogen.

Erythrocyte chloride. Calculation of the intracellular chloride of erythrocytes was made from the data of Bernstein (8) which give the chloride concentration of the rat red cell water, and from the data of Wang and Hegsted (9) which define the blood volume and hematocrit. According to the latter authors, the blood volume of a 220-gram rat closely approximates 7 per cent of the body weight and the hematocrit averages 45. Red cell water was taken as 72 grams per 100 ml. and the chloride concentration of red cell water as 72 mEq. per L. (8). Using these values, the chloride intracellular in erythrocytes may be calculated as 1.86 mEq. per Kg. of body weight. In a 220-gram rat the value would be 0.41 mEq.

Chloride intracellular in muscle and liver. Based on the studies of Cotlove (7) comparing the distribution volumes of inulin and chloride in

muscle after prolonged inulin infusion, the assumption has been made that chloride is not present in muscle cells. A similar assumption cannot be made for the liver. In this organ the volume of distribution of chloride exceeds that of sodium so that an intracellular position of chloride must be presumed. For lack of a better method, intracellular chloride in liver has been calculated on the assumption that the true extracellular volume of this organ is measured by the volume of distribution of sodium. In seven rats, the chloride space of liver exceeded the sodium space by 0.5 to 0.95 ml. corresponding to 0.066 to 0.120 mEq. of chloride. The average value in the 300-gram rat of 0.088 mEq. of intracellular chloride becomes 0.060 mEq. for a 220-gram rat.

Chloride within the lumen of the gastrointestinal tract. The chloride in the "transcellular" fluid within the gut was determined by flushing the intestinal tract with isotonic glucose solution. In five rats weighing approximately 300 grams, the chloride recovered averaged 0.22 mEq. and ranged from 0.14 to 0.28 mEq. Corrected to a body weight of 220 grams, the average value becomes 0.16 mEq. Several sources of error in this determination should be mentioned. Although the flushing procedure was carried out as rapidly as possible, the possibility cannot be excluded that some chloride diffused from the extracellular fluid through gut mucosa or serosa or through the incisions made in the gut wall. Also, the data were for use primarily in correcting the chloride space of viscera and to compare this space with the inulin space, as will be described below. Consequently the animals were not prepared in exactly the same manner as those used for carcass analysis. Food was withheld from those used for carcass analysis for only four hours while those used for the flushing procedure were starved for 24 hours. Although both groups had been on the same low residue diet, the differences in food intake might have produced differences in intraluminal gut chloride. It seems possible that these two sources of error, the one tending to give a falsely high value and the other a value falsely low, could balance out.

Chloride in cells of viscera exclusive of liver, brain, spleen and kidneys. Although chloride has been described as being intracellularly located in the pylorus, testes and lungs (2) the amounts present and their significance have not been clearly defined. The problem has been approached in the present study by determining the inulin, chloride and sodium spaces of gut, lung and testes after prolonged infusion of inulin. The data for these volumes are shown in Table IV. In this table the chloride and sodium spaces have been corrected for the amounts of these ions determined by

TABLE IV

Inulin and corrected chloride and sodium spaces of viscera after 6 and 24 hours of inulin infusion

Rat no.	Wt. of viscera *gm.*	Inulin space *ml.*	Inulin sp. / Viscera wt.	Corrected chloride space *ml.*	Corrected sodium space *ml.*	Inulin sp. / Cl space	Inulin sp. / Na space
			After 6 hours of inulin infusion				
2	21.4	5.35	.25	8.55	5.23	0.626	1.02
3	21.0	6.88	.33	6.21	4.95	1.11	1.39
4	23.5	7.23	.31	7.43	6.16	0.974	1.17
7	22.1	6.89	.31	8.62	7.92	0.80	0.87
10	20.5	6.56	.32	8.33	6.73	0.788	0.97
11	21.1	5.42	.26	6.57	5.42	8.826	1.00
12	21.1	6.26	.30	7.43	5.91	0.842	1.06
Averages			.29	7.59	6.04	0.852	1.07
			After 24 hours of inulin infusion				
5	22.6	6.11	.27	7.38	6.89	0.828	0.89
9	19.8	6.33	.32	6.06	5.78	1.04	1.10
13	23.7	9.93	.42	9.23	6.30	1.076	1.57
14	25.7	8.53	.33	7.48	6.84	1.19	1.25
Averages			.33	7.54	6.45	1.021	1.20
Averages (Both groups)				7.57	6.19		

the flushing experiments (see above) to be present in the gut lumen. This correction amounted to 0.22 mEq. of chloride and 0.43 mEq. of sodium for the 300-gram rats used.

At the outset it should be emphasized that use of the inulin space measurement for determining the amounts of sodium and chloride within the cells is hazardous. Although the measurement of inulin space by tissue analysis for inulin precludes errors in space measurement due to metabolism of inulin, it does not preclude error resulting from sequestration of inulin by macrophages as recently suggested by White and Rolf (21). These authors found that tissues rich in macrophages gave impossibly high inulin space values. With this source of error in mind, the present data can probably be interpreted as reliably by simple comparison of sodium and chloride spaces as by resorting to inulin space measurement. It can be seen that in every specimen the corrected chloride space exceeded the corrected sodium space, the difference averaging 1.38 ml. Assuming no sodium to be present in the cells and the sodium space to equal extracellular volume, the greater chloride space would represent chloride within the cells. The volume of 1.38 ml. is equivalent to approximately 0.17 mEq. of chloride or, corrected to a 220-gram rat, 0.12 mEq. In agreement with this interpretation is the inulin space at 6 hours. Inulin space at this time was in fair agreement with the sodium space but was smaller than the chloride space by 15 per cent. Taking the inulin space at 6 hours as approximating extracellular fluid volume, an intracellular chloride content similar to that defined above would be calculated. By this interpretation the further expansion of the inulin space at 24 hours to equality with the chloride space and to 33 per cent of the wet weight of the tissue would be ascribed to macrophage sequestration of inulin.

Disregarding the possibility of macrophage sequestration and using only the 24-hour values, the data could be alternatively interpreted as indicating that at 24 hours complete penetration of extracellular fluid by inulin had occurred. The identity of inulin and chloride spaces at this time would then indicate no chloride to be intracellular in viscera, a conclusion contrary to that of previous workers. Since this interpretation does not account for the smaller sodium space, the calculation based on the sodium space, indicating that 0.12 mEq. of chloride is present intracellularly in viscera, seems more reasonable.

Estimation of chloride space. As may be seen in Table II the chloride which must be considered as outside the extracellular fluid amounts, in a 220-gram rat, to 0.85 mEq. or 12.7 per cent of body chloride. Of this non-extracellular chloride, the greatest amounts are found in the red cells and in the transcellular fluid in the lumen of the gastrointestinal tract. Because of the technical difficulties in assessing the position of chloride in brain, spleen and kidneys, these organs have not been included in the calculations. Their omission would appear to be of little consequence. In the rat the combined weight of these organs amounts to only 1.5 per cent of body weight and from the data of Manery and Hastings (2) it can be calculated that their chloride content amounts to only 2.2 per cent of total body chloride. Thus, even if a relatively large fraction of the chloride of these organs were intracellular it would constitute only a small fraction of the total body chloride.

For the 220-gram rat, the chloride in extracellular fluid amounts to 4.88 mEq. (Table II). The value for serum chloride concentration used in calculating the chloride space was determined by analysis of the sera of 37 normal rats. The average was 109.5 mEq. per L. with a range from 105.6 to 113.6. Correcting the average value for serum water and for the Donnan factor gives a value of 124 mEq. per L. for the serum ultrafiltrate concentration and a chloride space of 47.4 ml.

Extracellular space calculated from extracellular sodium

Extracellular sodium has been calculated as a means of verifying the accuracy of the extracellular volume predicted from the distribution of chloride. The calculated amounts of sodium in cells and bone in a 220-gram rat are shown in Table V and the calculations are described below.

Bone sodium. In five rats weighing approximately 220 grams the bone salt sodium: calcium ratio was found to average 0.0200 with a range of 0.0187 to 0.0205 (Table VI). Total bone calcium in a 220-gram rat, calculated from the regression equation shown in Table I, amounts to 111.7 mEq. Multiplying the bone salt sodium:

TABLE V

Calculation of sodium in extracellular fluid and extracellular fluid volume of a 220-gram rat (50 gram FFDS)

	mEq	Percent of Total
Sodium Outside Extracellular Fluid		
Bone salt sodium (from Na:Ca ratio for bone salt and total body Ca)	2.21	21.2
Sodium intracellular in muscle (from rat muscle analyses (6) and muscle mass (25))	0.60	5.8
Erythrocyte sodium (from sodium concentration of red cell water (8), blood volume and hematocrit (9), and red cell water content)	0.14	1.3
Sodium within lumen of gut (determined by flushing of gut)	0.32	3.1
Sodium in cells of viscera (from comparison of sodium and inulin spaces after 6 hrs. of inulin infusion)	0	0
Total	3.27	31.4
Total Body Sodium	10.43	
Sodium in Extracellular Fluid	7.16	68.6

Serum ultrafiltrate sodium concentration, 149.6 mEq/L.

Sodium space = $\frac{7.16}{.1496}$ = 47.9 ml.

calcium ratio by total body calcium gave an average value of 2.21 mEq. for total bone salt sodium.

The accuracy of the estimate of the bone salt sodium is of importance in the assessment of electrolyte distribution because of the large fraction of body sodium present in bone. Possible sources of error lie in the method of estimation of the sodium : calcium ratio and in the assumption inherent in the calculation that the sodium : calcium ratio of all bones is the same as that of the specimens of bone taken for analysis.[2] The general agreement of the sodium : calcium ratio of 0.0200 obtained in the present study with that of 0.0206 obtained by Bergstrom (22) by flame photometric determination of sodium suggests the absence of gross errors in analysis. The validity of the assumption that all bones have the same sodium : calcium ratio is more difficult to assess. From fragmentary data, however, the assumption in the main appears to be correct. Agna and Knowles (23) have found in nine human subjects that rib and iliac crest are almost identical in their sodium : calcium ratios. On the other hand, the skull contained an average of 7 per cent more sodium per unit of calcium than did rib or ilium. Appendicular skeleton was not analyzed.[3]

Muscle cell sodium. Based on the data for muscle analyses of 13 rats by Cotlove, Holliday, Schwartz, and Wallace (6) the sodium intracellular in muscle was taken as 0.6 mEq. per 100 grams of fat free tissue. The muscle mass of the rat was taken from the data of Caster, Poncelet, Simon, and Armstrong (25). These authors were able to measure not only the muscle mass that was easily dissected from the skeleton but also the fraction adhering after dissection. The latter fraction was determined either from the composition of the ash or from the determination of actomyosin. The muscle mass of 320 to 350-gram rats of good nutritional status was found by these authors to average 45.5 per cent of body weight. In a 220-gram rat the muscle mass would closely approximate 100 grams and would contain 0.6 mEq. per L. sodium in the intracellular space.

Erythrocyte sodium. After Bernstein (8), the sodium concentration of red cell water is taken as 28 mEq. per L. The value of 0.14 mEq. for total red cell sodium was calculated in the manner described for erythrocyte chloride.

Sodium in lumen of the gastrointestinal tract. The quantity of sodium in the transcellular fluid in the gastrointestinal tract was determined as for chloride by the flushing procedure. The sodium recovered averaged 0.43 mEq. with the range of 0.35 to 0.50. The amount in the 220-gram rat

[2] Knowing the weights and calcium content of the bone samples taken for analysis and the total body calcium, the fraction of total skeleton represented by the bone samples may be calculated. The bone sample weights in terms of fat free dry solid averaged 1.45 grams and their calcium content averaged 10.7 mEq. per gram FFDS. With an average total body calcium content in a 220-gram rat of 111.7 mEq. the total skeleton as FFDS may be calculated from the above as weighing 10.4 grams. The bone samples then represented on the average 14 per cent of the total skeleton.

[3] If in the rat the sodium : calcium ratio of the skull were greater than the rest of the skeleton by 5 per cent as appears to be the case in the human, the error resulting from neglect of this discrepancy in the present calculations would be minimal. The dried skull of a 220-gram rat weighs 1.42 grams (24). Assuming the calcium content of dried skull to be 10.7 mEq. per gram and a 5 per cent greater sodium : calcium ratio for skull, the bone sodium would be greater than that found in the present calculation by 0.015 mEq. This amount represents 0.02 per cent of the total body sodium.

TABLE VI

Average values and range for bone calcium, sodium and chloride content and the bone salt sodium : bone calcium ratio

No. of rats	Sodium *mEq./100 gm. FFDS*	Calcium *mEq./100 gm. FFDS*	Chloride *mEq./100 gm. FFDS*	Chloride space *ml./100 gm. FFDS*	Sodium in chloride space *mEq./100 gm. FFDS*	Bone salt sodium / Bone calcium
5	29.7 32.8–26.7	1,074.4 1,032–1,109	6.83 8.65–5.23	55.1	8.23	0.0200 0.0187–0.0205

would be 0.32 mEq. or slightly more than 3 per cent of total body sodium.

Sodium intracellular in viscera. As mentioned above the liver was considered to contain no intracellular sodium; the volume of distribution of this ion was considered as a measure of the extracellular space. In the rest of the viscera studied (lungs, gastrointestinal tract and testes) the comparison of the inulin with the sodium space was used to assess the amount of sodium located intracellularly. For the comparison, the sodium space was corrected for the amount of sodium found by the flushing procedure to be present in the gut lumen. The correction amounted to 0.43 mEq. As may be seen in Table IV the inulin space approximates the corrected sodium space after six hours of inulin infusion. The data have been interpreted to indicate that no sodium is present within the cells of the viscera.

Estimation of extracellular sodium space. Summing up these corrections in Table V, it is seen that the sodium outside the extracellular space in a 220-gram rat would be 3.27 mEq. or 31.4 per cent of total body sodium.

For calculation of the extracellular sodium space, the value for extracellular sodium (7.16 mEq.) has been divided by the average sodium concentration in a serum ultrafiltrate. Serum sodium concentration in 15 normal rats averaged 146.7 mEq. per L. and the calculated serum ultrafiltrate concentration, 149.6 mEq. per L. The resultant extracellular sodium space of 47.9 ml. is in good agreement with the extracellular chloride space of 47.4 ml.

Partitioning of body water, sodium, and potassium. Total body water in a 220-gram rat averaged 149.4 ml. (Table I). Assuming extracellular volume as 48 ml. the extracellular phase would constitute 32 per cent of total body water or 21.8 per cent of body weight.

The partitioning of sodium in the body and the net concentrations of body sodium, potassium and magnesium in intracellular water are shown in Figure 1. For the calculation of intracellular volume, the volume of fluid within the gut was determined on six rats of 220-gram weight. The average value was 3.6 ± 0.42 (SD)ml. Subtracting this value and that of 48 ml. for extracellular volume from total body water gives 97.8 ml. for the volume of intracellular water. Extracellular potassium in a 220-gram rat was calculated as 0.19 mEq. The net concentration of potassium in cell water calculated as shown in Figure 1, would be 146 mEq. per liter. Sodium concentration in cell water, similarly calculated, would be 7.4 mEq. per liter.

For the calculation of the magnesium concentration of cell water the data of Duckworth, Godden, and Warnock (26) have been used. These authors found the calcium : magnesium ratio of rat bone to be 41.8. From this ratio and from total body calcium it may be calculated that 2.68 mEq. of magnesium are in the bones of a 220-gram

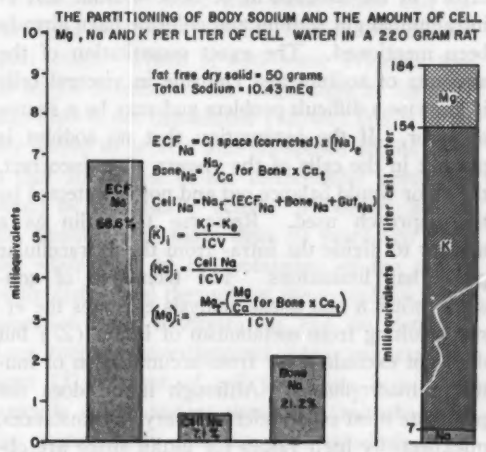

FIG. 1.

rat, leaving 3 mEq. in the soft tissues. Neglecting extracellular magnesium the cell water concentration of magnesium would be 30 mEq. per liter. This value agrees well with that of 33 mEq. per liter found by other investigators (6) for the magnesium concentration of muscle cell water. Similarly, from the calcium : phosphorus ratio for bone (26) and from total body calcium it can be calculated that 35.3 mMols of phosphorus (or 83 per cent of total body phosphorus) are present in bone.

DISCUSSION

For the precise interpretation of change in intracellular electrolyte during metabolic studies and for the proper partitioning of electrolyte in the body it is desirable that extracellular and intracellular volumes be defined. The present study has approached the problem of volume measurement by determining extracellular volume from extracellular chloride. An attempt has been made to verify the result by demonstrating that the sodium content of this extracellular volume corresponds in amount with that calculated from total body sodium minus the amounts present in bone and in cells. The good agreement of these independent approaches strongly suggests that extracellular volume has been correctly measured.

In spite of the good agreement, it should in all fairness be pointed out that errors could still exist which, if present on both the sodium and chloride sides of the balance, would cancel out. Possible errors in the assessment of bone sodium and of intraluminal gut sodium and chloride have already been mentioned. The exact quantitation of the amounts of sodium and chloride in visceral cells is likewise a difficult problem and may be a source of error. If the assumption that no sodium is present in the cells of the viscera were incorrect, the error would balance out and not be detected by the approach used. Recourse to inulin as a marker to divide the intra- from the extracellular phase has limitations. The technique of constant infusion and tissue analysis excludes the error resulting from metabolism of inulin (27) but does not exclude error from accumulation of inulin in macrophages. Although inulin does not penetrate most cells under ordinary circumstances, unexpectedly high values for inulin space are obtained in anuric patients (28) and in nephrectomized rats (21). Under conditions of nephrectomy, many substances normally extracellular in distribution predict volumes which are unreasonably high (29) and the possibility cannot be excluded that under these conditions ions or molecules penetrate the cellular phase. White and Rolf (21) using the nephrectomized rat and tissue analysis for inulin obtained a progressively large inulin space which, after 72 hours, predicted volumes exceeding total body water. Such findings may be related in some way to the removal of renal tissue, for the inulin space of rat muscle was significantly higher than that demonstrated by Cotlove (7) in animals with intact kidneys constantly infused with inulin. In White and Rolf's study (21), however, the anomaly was greatest in tissues rich in macrophages, such as liver, and the authors suggest that sequestration of inulin can occur in macrophage cells. In the non-nephrectomized animals in the present study it was found that in liver, the inulin space greatly exceeded both the sodium and chloride spaces after 24 hours of infusion. Hence, absence of renal tissue appears not to be the sole factor responsible for the large inulin spaces of nephrectomized animals. Whether in the other viscera studied, macrophage sequestration of inulin accounted for the increase in inulin space relative to sodium space as the inulin infusion was prolonged to 24 hours (Table IV) cannot be determined. This interpretation seems the most plausible, however, when the data are considered from all aspects, and the use of the six-hour inulin space, or the closely similar sodium space, as an approximation of extracellular volume appears to be justified.

A further source of error lies in the estimation of the "excess" chloride of connective tissue. It should be noted that the calculations assume that all of body collagen has an affinity for "excess" chloride equal to that of the large tendon masses taken for analysis. This assumption is probably in the main correct, as pointed out by Manery, Danielson, and Hastings (1), insofar as the connective tissue of muscle, which accounts for the majority of body collagen, is a direct extension of and probably similar in structure to tendon. However, the collagen of bone matrix and in visceral organs could well differ from tendon in its affinity for chloride. Also the wide differences in the amount of excess chloride in connective tissue

found by various investigators should be borne in mind. Manery, Danielson, and Hastings (1) found the chloride concentration of connective tissue water in rabbits to average 8.4 mEq. per L. greater than the concentration in a serum ultrafiltrate, a value in good agreement with that of 6 mEq. per L. found in the present study. On the other hand, another group of workers (30) found the concentration in dogs to be approximately equal to that of a serum ultrafiltrate while more recently a concentration in connective tissue water greater by 56 mEq. per L. than that of the serum ultrafiltrate has been reported in rats (31). It is possible that some of these differences are attributable to difficulties inherent in the methods of chloride analysis (4); the loss of variable amounts of water at the time of dissection would appear not to be the sole responsible factor.

While it is obvious from the above that errors may be present, most of them would appear to be of relatively little significance to the present study. As it stands, the study predicts that 87 per cent of total body chloride is in the extracellular fluid. Of the 13 per cent located outside of this compartment, more than half is in the erythrocytes and in the gastrointestinal tract. Because the amounts in cells and "excess" in connective tissue are so small, even gross errors in the quantitation of these fractions would have little effect on the overall assessment. For example, doubling the amount of "excess" chloride associated with connective tissue would reduce the present estimate of extracellular chloride by only 1.5 per cent. The conclusion that most of body chloride is in extracellular fluid appears justified. The data do not support the contention of other investigators employing differing techniques (32, 33) that 30 per cent or more of body chloride resides outside the extracellular fluid.

Previously, Manery and Hastings (2), Manery and Haege (34) and Amberson, Nash, Mulder, and Binns (35) have produced considerable evidence for the contention that chloride is predominantly extracellular in various tissues. At the same time these studies have suggested that intracellular chloride exists in pyloric tissue, stomach fundus and testes.

It would seem of importance that the exchangeable chloride of the rat measured with bromide over a 3-hour period closely predicts the true carcass chloride (4). A corollary of the findings presented is that the bromide or radio chloride space corrected for red cell bromide or chloride gives a close approximation of all phases of the extracellular fluid volume in the normal subject.

The value of 146 mEq. per L. obtained in the present study for the potassium concentration of cell water agrees well with the value of 140 mEq. per L. obtained by Harrison, Darrow, and Yannet (36) for the dog by carcass analysis using the uncorrected chloride space as the measure of extracellular volume. By indirect methods, however, widely diverging values have been obtained. Using inulin, D_2O, K^{42} and Na^{24} the intracellular potassium and sodium concentration of the dog averaged 115 and 35 mEq. per liter of cell water, respectively (37). These concentrations are greater for sodium and lower for potassium, when comparison is made with the present data. Possibly inulin under the conditions of these dog experiments under-estimates the total extracellular volume (38). Moore (39) using thiocyanate as a measure of extracellular volume and D_2O, K^{42} and Na^{24} to measure total water, exchangeable potassium and sodium, respectively, predicted in man an intracellular potassium concentration of 163 mEq. per liter of cell water. No estimate of transcellular fluid was possible in this study. This potassium concentration would seem to be high as a result of the fact that thiocyanate over-estimates the extracellular space (40, 41).

Evidence is at hand that intracellular and extracellular osmolar concentrations in the rat are equal (42). There is also some evidence that the major fraction of the magnesium of intracellular fluid is not dissociated (43, 44) and is probably bound to protein and phosphate anions (44) so that almost all of the intracellular potassium should be osmotically active. However, Macallum demonstrated more than 50 years ago (45) that while most of the potassium of the cell is evenly distributed throughout the cytoplasm, local points of high concentration can be detected. More modern investigations (46) suggest that about 13 per cent of cell potassium is not osmotically active and is present in mitochondria. The present study does not allow conclusions regarding the tonicity of cell fluid.

SUMMARY

An attempt has been made to determine the amount of sodium and chloride in the extracellular fluid in the rat by correcting total body sodium and chloride for the amounts of these ions which are outside the extracellular fluid. Corrections for chloride included the "excess" chloride of connective tissue calculated from total body collagen and the results of tendon analysis, chloride intracellular in liver calculated from the sodium space of liver, and chloride in the lumen of the gut determined from analysis of gut contents. Erythrocyte chloride was calculated from data in the literature. The intracellular chloride of the respiratory and gastrointestinal tracts and in the testes was assessed by tissue analysis after the constant infusion of inulin. From data of other investigators, it was concluded that no chloride is present intracellularly in muscle.

It was found that 87.3 per cent of body chloride can be considered as present in extracellular fluid. The chloride in erythrocytes and in the gut lumen, representing 6.1 and 2.4 per cent of total body chloride, respectively, account for the bulk of the non-extracellular chloride.

To obtain extracellular sodium, total body sodium was corrected for bone salt sodium, calculated as the product of the Na/Ca ratio for bone and total body calcium, and for sodium in the lumen of the gut, determined from the analysis of gut contents. Data in the literature were used for the calculation of erythrocyte sodium and for the intracellular sodium of muscle.

For a 220-gram rat, extracellular fluid volume calculated from chloride was found to be 47.4 ml. as compared with 47.9 ml. calculated from sodium. The good agreement between these values suggests that this approach and the corrections used are valid. Extracellular volume would thus represent 21.8 per cent of body weight or 32 per cent of body water.

From the data for extracellular volume and from other parameters of body composition obtained from carcass analysis and from the literature, the theoretical net concentrations of potassium, sodium and magnesium in cell water have been calculated.

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INCORPORATION OF N^{15}-L-ASPARTIC ACID INTO THE ABNORMAL SERUM AND URINE PROTEINS OF MULTIPLE MYELOMA (STUDIES OF THE INTER-RELATIONSHIP OF THESE PROTEINS)[1]

By ELLIOTT F. OSSERMAN, ADA GRAFF, MARGARET MARSHALL, DOLORES LAWLOR, AND SAMUEL GRAFF

(From the Departments of Medicine and Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N. Y., and the Medical Service and Biochemistry Division, Francis Delafield Hospital, New York, N. Y.)

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In a consideration of the abnormalities of protein metabolism observed in multiple myeloma, a number of fundamental problems remain unsolved. To state just two of these, it remains to be determined (a) precisely what relationship the abnormal serum globulins of myeloma bear to normal serum gamma globulin, and (b) what is the nature of the possible inter-relationship between the abnormal serum protein and urinary (Bence-Jones) proteins of myeloma when both are present in a particular case. The electrophoretic homogeneity of these abnormal serum and urinary proteins constitutes one of their major physicochemical features. Using this criterion of electrophoretic homogeneity to identify these abnormal proteins, it has been found (1–3) that approximately one-half of a group of one hundred myeloma patients has both a serum and a urine protein abnormality; another one-third of these cases shows only a serum abnormality, with no characteristic proteinuria, and the remaining one-sixth of the cases exhibits a discrete urine protein peak with no abnormal protein peak demonstrable in the serum.

The present study was designed to elucidate the inter-relationship between the serum and urine proteins in the first group of cases, *i.e.*, in those patients with both a serum and a urine abnormality. For this purpose, an isotopically-labelled amino acid was administered to a patient with multiple myeloma and the incorporation and turnover of this label was followed in these two proteins (Ms hereinafter designates the abnormal serum globulin, and Mu the urinary [Bence-Jones] protein). If the time-characteristics of the isotope curves obtained from Ms and Mu satisfied the criteria for a precursor-product relationship (Ms as precursor, Mu as product), it would be supportive, although not conclusive evidence for such an inter-relationship.

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SUBJECT AND METHODS

Case history

The subject (I. A.) was a 50-year-old colored female, admitted to the Delafield Hospital in April, 1954, with a ten-month history of increasingly severe low back pain. Past medical history was non-contributory. In 1951, she was found to have an anemia, the origin of which was obscure. Because of back pain she had been admitted to another hospital in March, 1954, where work-up had revealed: Hgb. 6.2 Gm.; Bence-Jones proteinuria; blood urea nitrogen, 10.7 mg. per cent; total serum protein, 10.8 Gm. per cent; A/G, 4.2/6.6; myeloma cells in bone marrow; and x-ray evidence of a pathological fracture of the third lumbar vertebral body.

When transferred to the Delafield Hospital one month later, laboratory investigation disclosed: Hgb. 4.9 Gm.; wbc 2,800, neutrophils 55 (15–40), lymphocytes 32, eosinophils 3, monocytes 10; platelets 164,000; non-protein nitrogen 23 mg. per cent; ESR 160 mm. per hr. Paper electrophoresis of the serum and urine (Figure 1) confirmed the presence of an electrophoretically homogeneous abnormal protein in both the serum and urine. The serum component (Ms) had the electrophoretic mobility of a γ-globulin; Mu was of beta mobility. Ms, when stained for carbohydrate by the periodic acid-Schiff technique (4), gave a strongly positive reaction, whereas Mu was Schiff-negative (Figure 1). Iliac bone marrow aspiration revealed 50 per cent myeloma cells. Widespread osteoporosis and osteolytic lesions were seen on skeletal survey.

Transient symptomatic benefit was obtained from a course of radiotherapy to the lumbar spine and transfusions in May, 1954, but there was roentgenographic evidence of overall disease progression. A further palliative course of 1,000 r. to the thoracic spine was administered in early September, 1954. No urethane or other chemotherapeutic agent was administered prior to the isotope study. Despite two transfusions in the two weeks pre-

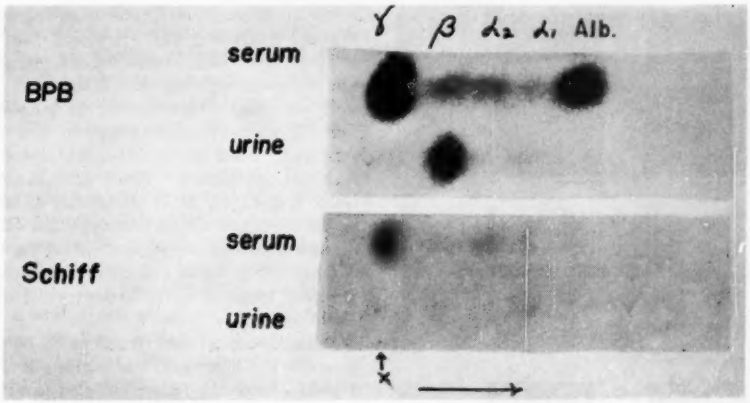

FIG. 1. PAPER ELECTROPHORETIC PATTERNS OF THE SERUM AND URINE OF PATIENT I. A., STAINED FOR PROTEIN WITH BROMPHENOL BLUE (BPB) AND FOR CARBOHYDRATE WITH THE SCHIFF TECHNIQUE

"X" indicates the site of sample application, and the arrow designates the direction of migration.

ceding the study, the blood count on the day of isotope administration was: Hgb., 7.0 Gm.; rbc, 2.47 million; wbc, 2,500; platelets 88,000. Further data at this time (9/24/54): Non-protein nitrogen, 43 mg. per cent; uric acid, 4.1 mg. per cent; calcium, 12.8 mg. per cent.

The isotope study was well tolerated, and, throughout the 17-day observation period, appetite and food intake were excellent. An intercurrent cystitis secondary to the presence of an indwelling catheter responded satisfactorily to antibiotics. The effect of this cystitis on the qualitative nature of the proteinuria, and the procedural changes necessary for urine protein separations, are described below. Three transfusions of packed red blood cells from 500 cc. of whole blood were given during the 17-day period.

Following the isotope study, a therapeutic trial of cortisone and subsequently a course of urethane were administered with slight subjective (but no objective) evidence of benefit. Urethane was discontinued after 6 weeks (total dose 105 Gm.) because of pancytopenia. Two weeks prior to death, the patient developed nitrogen retention, and despite supportive measures she expired on 28 December 1954.

Autopsy confirmed the widespread osseous destruction with myeloma tissue. The kidneys showed the tubular epithelial degenerative changes and the proteinaceous casts considered typical for Bence-Jones protein damage. Fine vacuolization of the tubular epithelial cells was also seen. The immediate cause of death appeared to have been an extensive bilateral lobular pneumonia.

Physico-chemical characteristics of the subject's myeloma serum (Ms) and urinary (Mu) proteins

Figure 2 shows the moving boundary electrophoretic patterns of the whole serum, and Figure 3, the electrophoretic and ultracentrifugal diagrams of the isolated serum (Ms) and urine (Mu) proteins. Throughout the experimental period, the total serum protein concentration remained at 14 Gm. per cent with the following percentage distribution of components: albumin 15.9, alpha-1 globulin 2.4, alpha-2 globulin 4.9, beta-globulin 6.1, gamma-globulin (Ms) 70.7 per cent. Thus, the Ms component was present at a concentration of approximately 10 Gm. per cent. The electrophoretic mobility of Ms in Veronal buffer, pH 8.6, ionic strength 0.1, was 1.3×10^{-5} $cm.^{-2}$ $sec.^{-1}$ $volt^{-1}$. The sedimentation constant $S_{20,w}$ of Ms = 6.0 S. Mu had a mobility of 2.7×10^{-5} $cm.^{-2}$ $sec.^{-1}$ $volt^{-1}$, and a sedimentation constant $S_{20,w}$ of 3.5 S. The mono-dispersity of these proteins is apparent from the

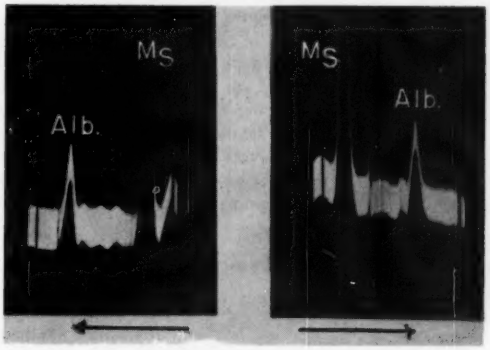

FIG. 2. ELECTROPHORETIC PATTERNS, ASCENDING (LEFT) AND DESCENDING (RIGHT) BOUNDARIES OF WHOLE SERUM OF SUBJECT I. A., OBTAINED IN VERONAL BUFFER, PH 8.6

The arrows indicate the direction of migration.

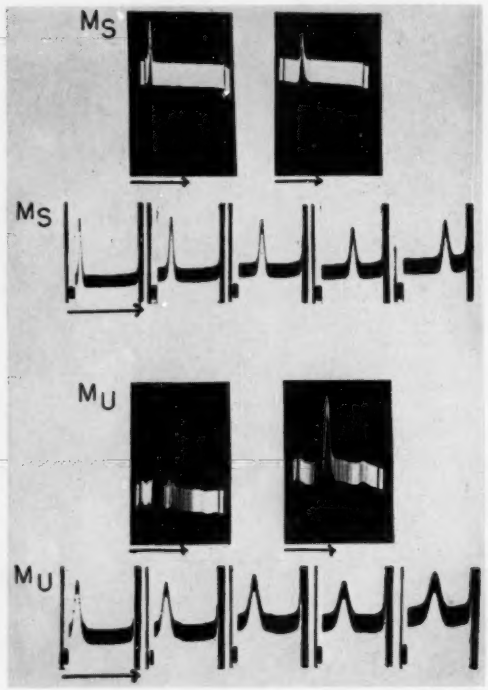

FIG. 3. ELECTROPHORETIC AND ULTRACENTRIFUGAL PATTERNS OF MS AND MU

The electrophoretic patterns were obtained in Veronal buffer, pH 8.6, and descending boundaries are shown. For Ms, the electrophoretic patterns were photographed at 15 and 85-minute intervals; for Mu, at 38 and 80 minutes. The arrows indicate the direction of migration.

In the ultracentrifugal patterns, sedimentation proceeds to the right as indicated by the arrows, and all photographs were taken at 16-minute intervals.

electrophoretic and ultracentrifugal diagrams. End amino group analysis[2] of Ms disclosed: glutamic acid, 1.61 M/160,000 Gm. protein; and phenylalanine, 1.40 M/160,000 Gm. protein. It is noteworthy that this is the only myeloma protein of over 20 studied thus far by Dr. Putnam in which phenylalanine has been found as an amino end group. N-terminal amino acid analysis of Mu was not carried out.

Experimental protocol

N^{15}-L-aspartic acid ($COOH \cdot CH_2 \cdot CH \cdot N^{15}H_2COOH$) was prepared from fumaric acid and ammonia containing N^{15} in the presence of *E. coli* according to the method of Wu and Rittenberg (5). A single dose of 10.6 Gm. of N^{15}-L-aspartic acid, containing 31 per cent excess N^{15} was administered along with 2 Gm. of $NaHCO_3$ immediately preceding a breakfast of eggs, toast, milk and coffee. No untoward gastrointestinal side-effects were experienced. Food intake throughout the experimental period was satisfactory. Blood samples were obtained at 1, 2.5, 4, 8, 12, 16, 20, 24, 30, 36 and 48 hours, twice daily on the third, fourth and fifth days, and daily thereafter to the termination of the experiment on the seventeenth day. An indwelling bladder catheter permitted complete and precisely timed urine collections. All stools were collected for 6 days.

Serum proteins were fractionated by dilution with 3 volumes of 0.1 M NaCl and dialysis at 2 to 3°C against 1.64 M ammonium sulfate buffered to pH 7.2 as outlined by Grisolia and Cohen (6). The abnormal myeloma globulin (Ms) precipitates were washed twice with 1.64 M ammonium sulfate dissolved in 0.15 M NaCl and re-precipitated with 5 per cent trichloracetic acid (TCA). The proteins in the supernate from the ammonium sulfate dialysis, comprising principally albumin, with approximately 15 per cent alpha and beta globulins, were precipitated with 5 per cent TCA. This fraction is hereinafter designated Alb. (α, β) in recognition of its residual contamination with these alpha and beta globulin constituents. All TCA-precipitated proteins were washed a minimum of five times with 5 per cent TCA to remove non-protein nitrogenous constituents.

The urinary protein preceding the experiment and during the first two days of the study consisted almost exclusively of the abnormal, electrophoretically homogeneous, Mu component. Accordingly, the urine samples during this initial period were half-saturated with ammonium sulfate, and the precipitated protein re-dissolved in water, precipitated with 5 per cent TCA, dissolved again in 0.75 M NaOH, re-precipitated with TCA, and washed five times with 5 per cent TCA. With the development of the intercurrent cystitis on the second day of the study, there was gross hematuria and contamination of Mu with blood protein components. It thus became necessary to carry out an extensive fractionation procedure on the urine samples subsequent to the second day, as follows: (a) urine acidified to pH 5.5 and half-saturated with ammonium sulfate; (b) the resultant precipitate dissolved in water, followed by a 48-hour water dialysis (at 10°C) which precipitated hemoglobin and small amounts of other globulins, leaving Ms, Mu and albumin in solution; (c) precipitation and separation of Ms globulin by dialysis against 1.64 M ammonium sulfate at pH 7.2; (d) removal of ammonium sulfate in the supernate by water dialysis; (e) concentration by osmodialysis against 25 per cent polyvinylpyrollidone; (f) precipitation of Mu and albumin with 5 per cent TCA; and (g) washing five times in 5 per cent TCA. Electrophoretic analysis of these samples after step (e), *i.e.*, after separation of Ms and prior to TCA precipitation, demonstrated them to be comprised of 85 per cent of Mu, and 15 per cent

[2] This N-terminal amino group analysis was kindly performed by Dr. Frank W. Putnam, formerly of the Department of Biochemistry, University of Chicago, and now Professor of Biochemistry, University of Florida College of Medicine, Gainesville, Florida.

albumin and alpha globulins, the latter representing the extent of residual contamination with serum protein constituents. Further purification of these urine protein samples could not be carried out due to the limitations of sample size. It will be seen that this complication did not appreciably alter the form of the Mu isotope concentration curve.

Glutamic and aspartic acids were isolated from the TCA-precipitated proteins after hydrolysis with 6 N HCl using the basic polyamine, formaldehyde resin (Amberlite IR-4) according to the technique described by Cannan (7). Urea (both serum and urine) was isolated as the di-xanthydryl-urea derivative. Urinary ammonia was liberated with saturated potassium carbonate and transferred by aeration into boric acid. The Kjeldahl method was employed for all nitrogen determinations. N^{15} abundance was determined with the mass spectrometer.

RESULTS

Over 70 per cent of the administered N^{15} was excreted in the urine as urea nitrogen within the first three days. Another 6 per cent appeared as urinary urea nitrogen between the third and the eighth days. Urinary ammonia nitrogen accounted for only one per cent of the N^{15} loss, and total stool nitrogen for another 4.8 per cent. Thus, over 80 per cent of the administered label was excreted as non-protein nitrogen.

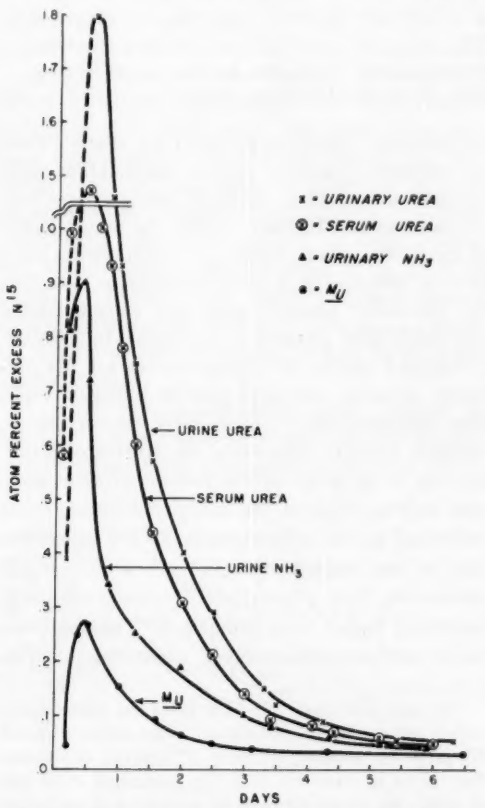

FIG. 4. N^{15} ABUNDANCE IN SERUM AND URINARY UREA NITROGEN, URINARY AMMONIA AND THE MYELOMA URINARY (MU) PROTEIN

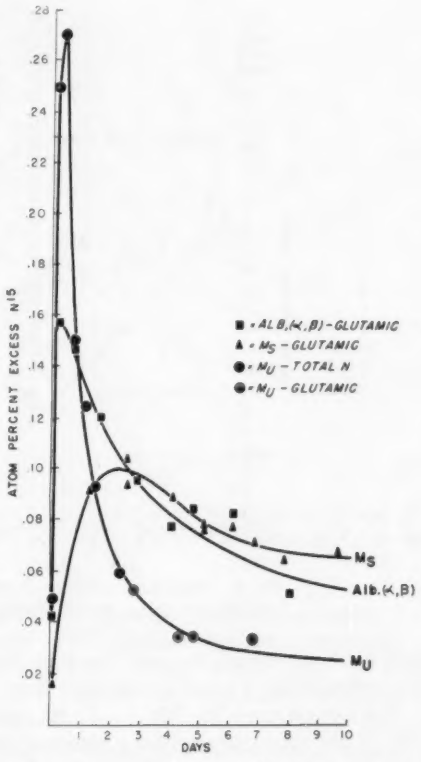

FIG. 5. N^{15} ABUNDANCE IN ▲ MS-GLUTAMIC ACID, ■ ALB. (α, β)-GLUTAMIC ACID, ● MU TOTAL NITROGEN AND ⊙ MU GLUTAMIC ACID

The isotope abundance curves for serum and urinary urea, urinary ammonia and for the Mu protein are shown in Figure 4. The Mu curve is seen to be essentially similar in form and time-characteristics to the urea and ammonia curves, indicating that the metabolic turnover of this abnormal protein proceeds at a rate comparable to that of the non-protein nitrogenous substances.

The isotope curves for Ms, Alb. (α, β), and Mu are shown in Figure 5. By contrast with the rapid incorporation and decline of isotope in Mu, the maximal isotope concentration in Ms was not

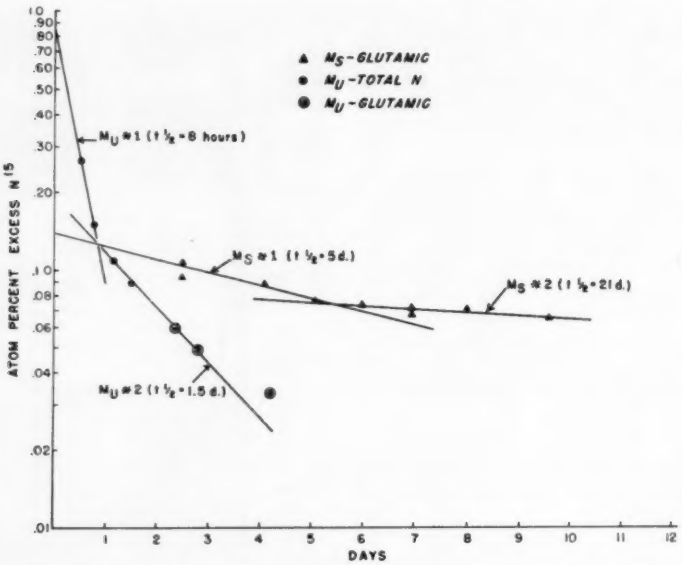

FIG. 6. SEMI-LOGARITHMIC PLOT OF N^{15} ABUNDANCE DATA FOR ▲ MS-GLUTAMIC ACID, ● MU-TOTAL NITROGEN AND ⊙ MU-GLUTAMIC ACID

reached until the second day, and the subsequent decline proceeded at a much slower pace than for Mu. The isotope curve for Alb. (α, β) was intermediate in its time-characteristics between these two myeloma protein curves, showing faster rate of decline than Ms, but considerably slower than Mu.

Semi-logarithmic plots of the declining portions of the Ms and Mu isotope curves are shown in Figure 6. In both instances, the data appear to show the best fit to two separate exponential functions. For Ms, the first exponential (E^1) has a $t\frac{1}{2}$ of 5 days; the second (E^2), a $t\frac{1}{2}$ of 21 days. For Mu, (E^1) has a $t\frac{1}{2}$ of 8 hours, and (E^2), of 1.5 days. The Alb. (α, β) isotope curve (not shown in Figure 6) follows a similar double exponential decline pattern, with the $t\frac{1}{2}$ of (E^1) equal to 3.5 days; $t\frac{1}{2}$ of (E^2) equal to 12.5 days.

The initial rapid rates of decline in isotope concentration in these three proteins (the E^1s) undoubtedly represent the net resultant of several functions of isotope distribution and dilution into amino acid and protein pools of different magnitudes proceeding concomitantly with protein degradation and excretion (Mu). The fact that single exponential functions approximate the data during these early periods is probably fortuitous. The second decay functions should more nearly reflect the respective rates of protein degradation, with the probable implicit error of isotope recycling. In the case of Mu, there exists the added factor of excretion. The extent to which the isotopic label is liberated by protein breakdown back into the metabolic nitrogen pool and reincorporated into these same proteins is impossible to estimate.

Figure 7 shows the isotope curves for Ms glutamic, aspartic and total protein nitrogen. The time-characteristics of these three curves are essentially similar, supporting the postulate of uniformity of turnover of the formed protein molecule, and, as expected, the isotope abundance in the individual amino acids exceeds the N^{15} concentration in total protein nitrogen. It is particularly noteworthy that glutamic N^{15} concentration was uniformly higher than aspartic N^{15}, although aspartic had been administered.[3] This concentration

[3] Wu and Rittenberg (5) have noted the same concentration differential, *i.e.*, plasma and tissue protein glutamic N^{15} exceeding aspartic N^{15} after N^{15}-aspartic administration. This is consistent with the recognized rapid rate at which the amino nitrogen of aspartic acid exchanges with the nitrogen of the metabolic pool. Aspartic acid is so rapidly deaminated that its amino group may be considered to behave metabolically like ammonia.

difference for glutamic, aspartic and total protein nitrogen was also found in the Alb. (α, β) samples (Figure 8).

The Mu samples during the first 48 hours were too small to permit isolation of glutamic and aspartic acids, and only total protein N^{15} could be measured. When the pooled Mu samples of the third and subsequent days were available for glutamic and aspartic isolations, these were performed. As indicated in Figures 4, 5, and 6, the glutamic N^{15} values appeared to follow the curve of the earlier Mu-total N points. In these later Mu samples, the glutamic N^{15} values and the total Mu protein N^{15} values were not significantly different within the limits of accuracy of the analytic methods.

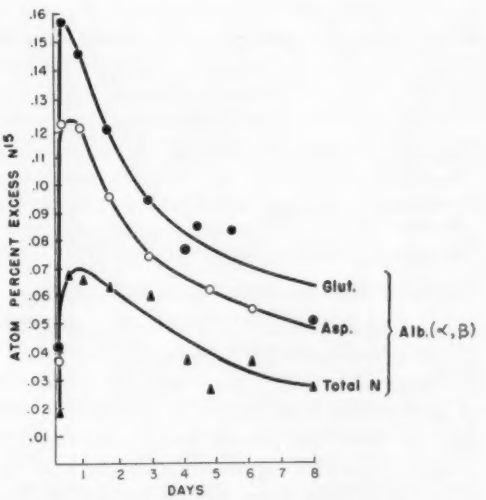

FIG. 8. N^{15} ABUNDANCE IN THE ● GLUTAMIC, ○ ASPARTIC AND TOTAL PROTEIN NITROGEN ▲ FRACTIONS OF ALB. (α, β)

DISCUSSION

A number of physico-chemical studies of myeloma serum and urinary proteins (8–10) have documented a consistent difference in molecular weights of these two constituents. Although the serum globulins vary considerably in molecular weight in individual cases, they most frequently have been found to have sedimentation and diffusion constants indicative of molecular weights in the range of 160,000. The urinary Bence-Jones proteins, by contrast, are usually of much smaller size, with mean molecular weights in the range of 35,000 to 40,000. The sedimentation constants of Ms and Mu of the patient in this present study are consistent with molecular weights in these respective ranges.

A study (4) of the carbohydrate content of myeloma serum and urine proteins in our laboratory indicated that, whereas the myeloma serum globulins contained a significant quantity of conjugated Schiff-positive material, presumably protein-bound hexose and hexosamine, the urinary myeloma proteins were apparently devoid of these substances. These observations were subject to at least three possible interpretations, namely (I) that Ms and Mu are two distinct protein moieties, independently elaborated, and independently metabolized, (II) that the urinary protein (Mu) represents a fragment of the serum globulin (Ms) after removal of the conjugated carbohydrate moiety, or (III) that the protein excreted in the urine represents a portion of a larger body pool of this material which is functioning as a precursor of the serum globulin, *i.e.*, prior to conjugation with its carbohydrate moiety.

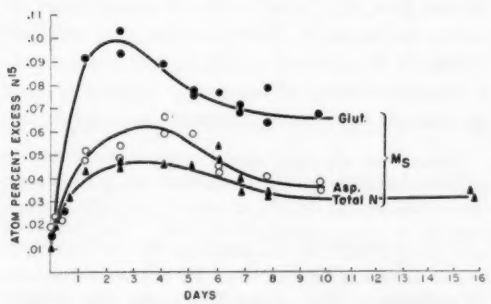

FIG. 7. N^{15} ABUNDANCE IN THE ● GLUTAMIC, ○ ASPARTIC AND TOTAL PROTEIN NITROGEN ▲ FRACTIONS OF MS

If the second postulate were correct, *i.e.*, Ms is the precursor of Mu, the isotope concentration maximum in Ms should have preceded and exceeded the isotope maximum in Mu. Since just the opposite relationships were observed between these two turnover curves, postulate II would appear to be invalid, leaving the choice between I and III. As will be seen shortly, after consideration of other available data, this choice cannot, as yet, be clearly and conclusively made.

Essentially similar results to those herein reported have been obtained by Putnam, Hardy,

TABLE I

	Labelled compound	Serum globulin		Urinary (Bence-Jones) protein		Albumin
		S_{20}	$T_{\frac{1}{2}}$	S_{20}	$T_{\frac{1}{2}}$	$T_{\frac{1}{2}}$
Putnam and Hardy (11)	$NH_2 \cdot CH_2 \cdot C^{13}OOH$	6.2 : 8.8	17–20 d.	3.08	18 hours	No data
Hardy and Putnam (12)	$N^{15}H_2 \cdot CH_2 \cdot COOH$	6.6 (Cryo-)	? about 20 d.	2.14	1.8 d.	No data
This paper	$HOOC \cdot CH_2 \cdot CH \cdot N^{15}H_2COOH$	6.0	E^1 = 5 d. E^2 = 21 d.	3.5	E^1 = 8 hrs. E^2 = 1.5 d.	E^1 = 3.5 d. E^2 = 12.5 d.

Meyer, and Miyake (11–13) in studies using C^{13}-glycine in one patient, N^{15}-glycine in a second, and DL glutamic acid-$1C^{14}$ in a third. A comparison of their results, from studies (11) and (12) with the data from the present study, is outlined in Table I. Several significant differences in the protocols of these three studies are notable, *viz.*, Putnam and Hardy's first subject (11) was receiving urethane throughout the experimental period; the subject of the second study had evidence of advanced renal damage and nitrogen retention, and had also received urethane just prior to the isotope experiment. This patient's serum protein was a cryoglobulin, with only aspartic acid in the N-terminal position. The subject of the study herein reported was selected because of the relatively typical nature of the abnormal serum and urine proteins exhibited, and the apparent freedom from the possible effect of prior therapy or renal damage. Despite these differences, and the fact that different amino acids were employed, the observed results in these three studies are in essential agreement with regard to the turnover rates of the myeloma proteins. It must be recognized that the biosynthetic labelling procedure employed in this study and in the studies of Putnam and his co-workers has, implicit within it, a serious source of error due to reincorporation of the isotope. This factor of recycling is primarily operative in the later time periods. Accordingly, the calculated half-lives must be considered as only very gross approximations to the true biological life-spans of these protein constituents. Within these clearly recognized limitations, however, one may speculate on the possible interpretations of the observed isotope turnover curves of Ms and Mu.

The extremely rapid turnover of Mu may in part be explained by the rapid sequestration of this protein in the urine, and, hence, its non-availability for later reincorporation of "recycling isotope." The approximately 15 per cent residual contamination of the later Mu samples with albumin and minimal amounts of alpha globulins due to the intercurrent cystitis might be expected to have introduced an error in the direction of prolonging the observed half-life of the urinary protein, since the serum Alb. (α, β) fraction displayed a much slower turnover rate than that found for Mu.

An isotope turnover study of the albumin in the urine of a patient with nephrosis, comparing the isotope half-life in urinary albumin with serum albumin of the same subject, would probably represent the best reference with which to evaluate the function of urinary sequestration on the form of the isotope turnover curve. Although turnover studies of serum proteins in nephrosis have been reported, the urinary proteins have not been examined.

The half-life of serum Alb. (α, β) as measured in our myeloma subject was approximately 12.5 days. This is considerably shorter than the values for albumin of normal subjects reported from other studies (14, 15) which utilized biosynthetic labelling techniques.[4] Thus, London (14) obtained a half-life for albumin of 20 days in normal subjects after feeding N^{15}-glycine. Masouredis and Beeckmans (15) found comparable albumin half-

[4] These data are more closely comparable to the experimental situation of this study than are the more extensive reports (15, 17, 18) of albumin half-life as measured by *in vitro* labelled I^{131}-albumin. The values for half-life of I^{131}-albumin are about one-half to one-fifth as long as the values for the biosynthetically labelled protein. This shorter life-span of the I^{131}-albumin may reflect a significant alteration (? partial denaturation) of the albumin molecule in the *in vitro* labelling process, resulting in a shortened life-span.

lives of 27.6 days in a patient with polycythemia vera, and 39.4 days in a subject with inactive rheumatic heart disease when they employed orally administered C^{14}-glycine. Volwiler, Goldsworthy, MacMartin, Wood, Mackay, and Fremont-Smith (16) report a range of 23 to 44 days in half-life for albumin in normal subjects when they used orally administered S^{35}-cystine. Whereas the rapid turnover of the Alb. (α, β) fraction in our myeloma subject may partly be due to the contamination of albumin with alpha and beta globulins of more rapid turnover rates than albumin itself, it is suggestive of a true augmentation in the rate of albumin degradation in this derangement of protein metabolism. The lack of data on patients with other neoplastic diseases and associated hypoalbuminemia, however, prevents any speculation regarding the specificity or significance of this phenomenon.

An extensive range of values for the half-life of normal gamma globulin has been reported from the several laboratories engaged in these studies. As in the case of other plasma protein fraction life-span studies, differences in labelling techniques and methods of protein fractionation appear to exert a profound influence on final calculated values of turnover rates. Thus, Volwiler and his co-workers (16) using orally administered S^{35}-cystine obtained gamma globulin half-lives of 48 to 85 days in normal subjects, and 31 days in a patient with cirrhosis. A half-life range from 19 to 60 days for this fraction has been reported by Armstrong and his associates (19, 20). The half-life value of approximately 21 days for the Ms protein herein reported, and the comparable values for the myeloma serum globulins reported by Putnam and Hardy (11, 12) are not clearly divergent from the available isotope turnover data for normal gamma globulin fractions, but again it must be stressed that the differences in labelling and fractionation techniques preclude any reliable comparisons.

In conclusion, it may be stated that the *relative* rates of turnover of the abnormal serum and urine proteins in a case of multiple myeloma have been studied after the oral administration of N^{15}-L-aspartic acid, in an effort to ascertain whether the urinary protein could be a product or a fragment of the larger molecular-sized serum constituent. Because the isotope concentration maximum of the urine protein (Mu) preceded and exceeded the isotope maximum in the serum globulin (Ms), the latter (Ms) cannot be construed to have been the precursor of the former (Mu). Having observed similar results in their isotope studies of those abnormal myeloma proteins, Putnam, Hardy, Meyer, and Miyake (11–13) have suggested that the opposite inter-relationship may exist, *i.e.*, that the Bence-Jones urinary proteins may represent precursors or abortive products of serum globulin synthesis. This alternative hypothesis is surely worthy of further consideration, but the present authors do not believe that the currently available experimental data justify this interpretation. It would appear that Mu is rapidly synthesized and rapidly excreted, whereas Ms is more slowly elaborated and degraded. The experimental evidence fails to establish that either protein is the precursor or product of the other.

SUMMARY

1. The rates of incorporation and degradation of serum albumin, the abnormal serum (Ms) and urine (Mu) proteins in a patient with multiple myeloma have been studied employing orally administered N^{15}-L-aspartic acid as the biosynthetic label.

2. The turnover rate of Mu was found to be extremely rapid, with a half-life of 1.5 days, whereas the myeloma serum globulin, Ms, was found to have a much slower turnover rate (half-life = 21 days).

3. The isotope concentration maximum in Mu preceded and exceeded the isotope maximum in Ms.

4. These data are interpreted as being incompatible with the hypothesis that the larger molecular-sized serum globulins of myeloma are precursors of the smaller urinary (Bence-Jones) proteins. They also fail to establish the opposite inter-relationship, *i.e.*, that the Bence-Jones proteins are "abortive precursors" of serum globulin synthesis.

5. Until further data prove to the contrary, the thesis that Ms and Mu are separate constituents, independently elaborated, would seem most readily acceptable.

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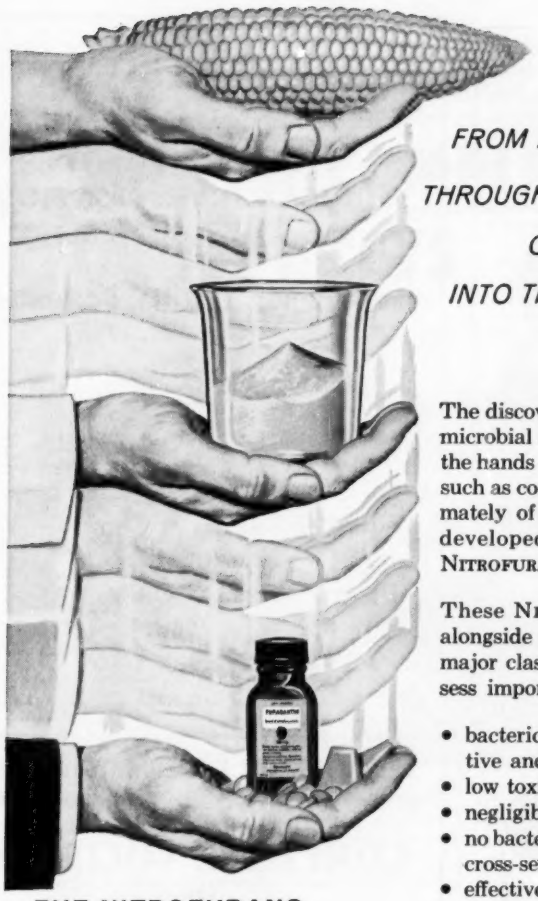

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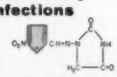

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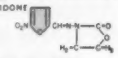

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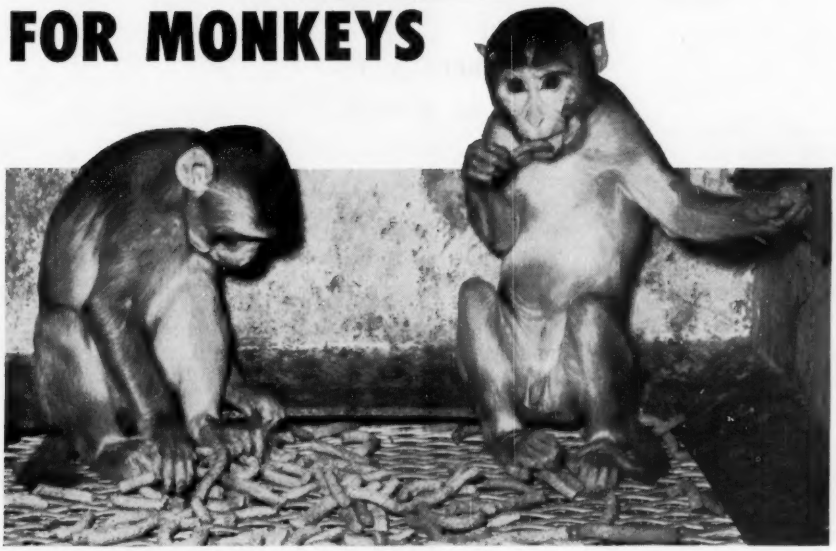

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FEBRUARY, 1957

Vol. II, No. 2

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A Usurper, the Virocyte. J. BERNARD and G. MATHÉ.

Original Articles

Studies on Glucagon. II. Similarities between the Anterior Pituitary and the Alpha Cells of the Pancreas in the Dog. J. STAHL and M. DORNER.

Pulmonary Alterations Following the Inhalation of 100% Oxygen in Normal Subjects and in Emphysematous Subjects. A. DE COSTER and H. DENOLIN.

Pathologic Physiology of the Albuminuria of Nephrosis and Amyloidosis. P. P. LAMBERT, F. GREGOIRE and C. MALMENDIER.

Immunochemical and Immuno-Electrophoretic Studies in Hyperglobulinemia. P. BURTIN, L. HARTMANN, J. HEREMANS, J. J. SCHEIDEGGER, F. WESTENDORP-BOERMAN, R. WIEME, CH. WUNDERLY, R. FAUVERT and P. GRABAR.

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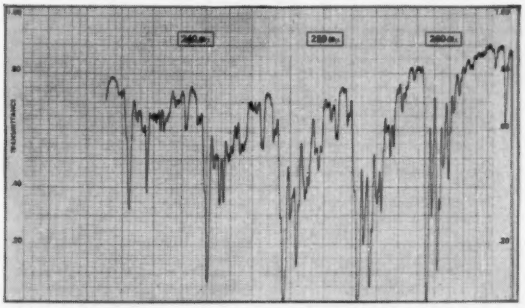

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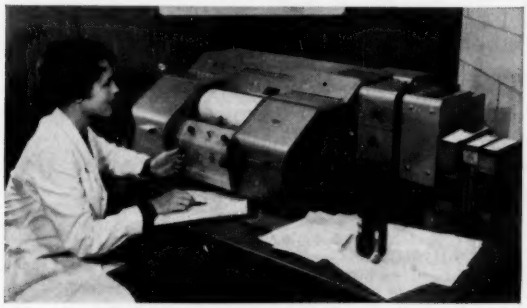

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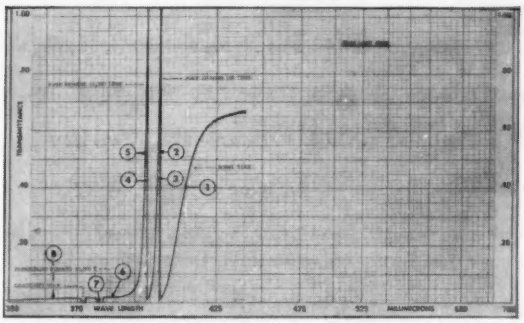

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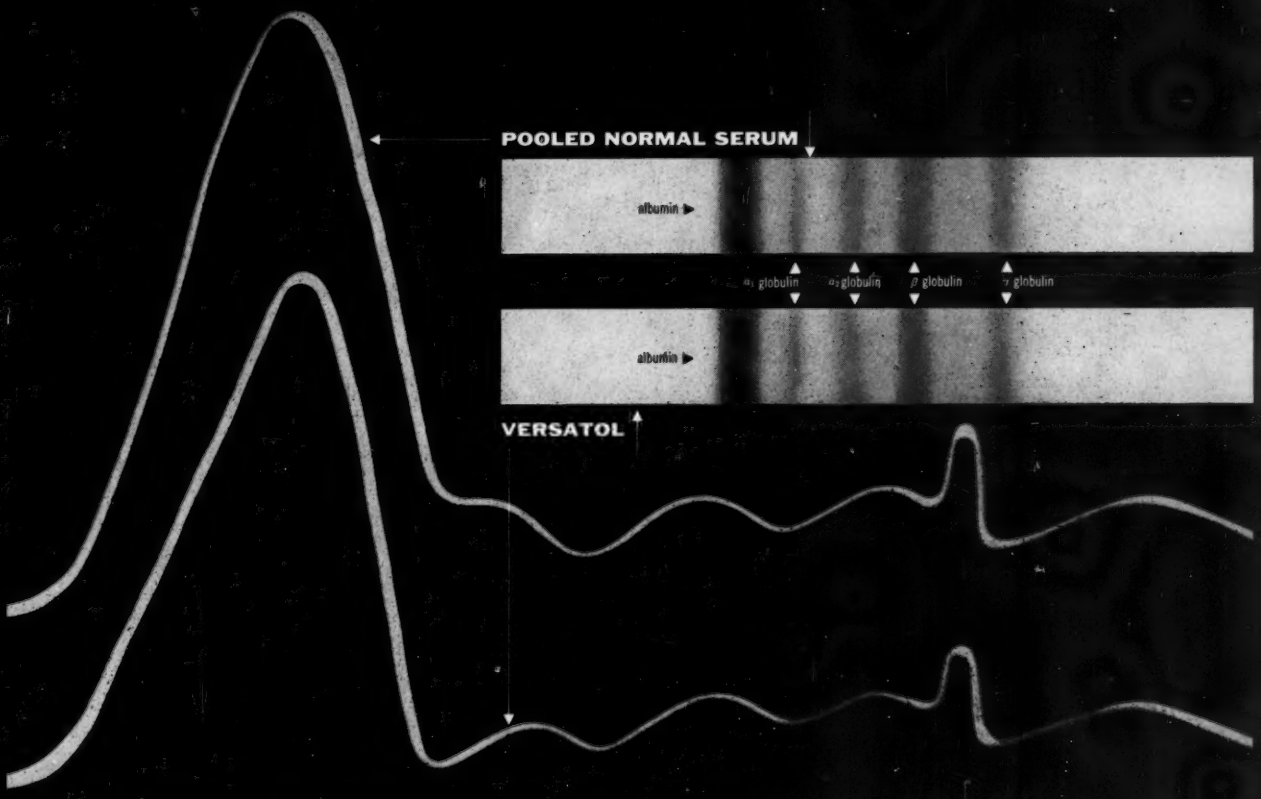

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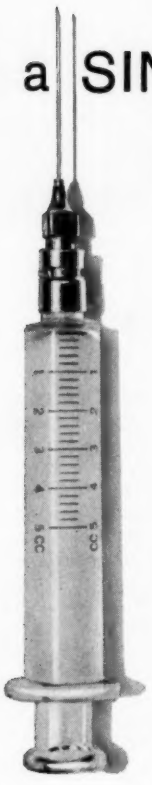

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